

C.

THE MECHANISM OF HÆMOLYSIS WITH SPECIAL
REFERENCE TO THE RELATIONS OF
ELECTROLYTES TO CELLS

BY
G. N. STEWART

*(From the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve
University)*

REPRINTED FROM

THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
VOL. I, No. 1. JUNE 1909

WELLCOME INSTITUTE LIBRARY	
Coll.	wellcome
Coll.	gen.
No.	QW 570
	1907
	385m



22500887528

THE MECHANISM OF HÆMOLYSIS WITH SPECIAL REFERENCE TO THE RELATIONS OF ELECTROLYTES TO CELLS

G. N. STEWART

(From the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve University)

(Received for publication March 26, 1909)

CONTENTS

Introduction.....	49
Part I. Physico-chemical properties of erythrocytes and their relation to the histological structure.....	57
Historical note	57
Condition of the intracorpuseular electrolytes.....	59
Osmotic pressure of erythrocytes and plasma	72
Cause of the low conductivity of the erythrocytes.....	73
Existence of an envelope in erythrocytes	74
Conditions governing the permeability of erythrocytes.....	79
"Membranes" and electromotive phenomena of tissues.....	83
Part II. The mechanism of hæmolysis	88
Hæmochromolysis and stromatolysis	88
Hæmolytic experiments on denatured erythrocytes	102
Permeability of the nucleus and its relation to hæmolysis	112
Classification of laking agents	114
Summary	120

INTRODUCTION

An enormous literature has gathered about the important subject of hæmolysis, but comparatively little attention has been given to the intimate mechanism of the process. It is, of course, of interest and importance to know that the serum of an animal of given species will lake the corpuscles of an animal of another given species; that a hæmolytic power not normally present in a given serum can be con-

ferred on it by immunization of the animal with alien corpuscles; that in some diseases the serum becomes lytic to its own corpuscles or to the corpuscles of the normal animal; that a great number of chemical substances will lase the erythrocytes of any animal, and so on. But it is not less interesting to inquire what happens in the erythrocyte under the influence of any hæmolytic agent when it is laked; why does the blood pigment pass out of it; what is the condition of the remainder of the corpuscle after it has lost its hæmoglobin; and very specially, whether the process is the same in all kinds of laking? This last inquiry has been surprisingly neglected. The tacit assumption has been made by the majority of writers that hæmolysis is hæmolysis however it is produced. To take one instance, it is curious to observe how it has been assumed by writers who have investigated the chemistry of the stromata that it is a matter of no consequence whether the blood-pigment has been got rid of by freezing and thawing, by grinding up the corpuscles with sand, by the copious addition of water, or by the action of ether and similar substances. Recently there have been symptoms that the mechanism of hæmolysis has excited more attention.

Liebermann¹ has brought forward evidence that in several forms of hæmolysis ("neutral guaiac saponin," soaps) the determining factor is the formation of a compound between stroma constituents and the hæmolytic substance.

G. Bayer² believes that the lecithin of the erythrocyte membrane is changed into a water-soluble condition by the bile-salts, and that in consequence of this the hæmogloberescapes. Proteins, according to him, may also be rendered soluble by bile salts.

Neuberg and Rosenberg³ and Neuberg and Reicher⁴ lay stress on the lipolytic power of certain agglutinating substances (croton, ricin) and of certain hæmolysins (cobra, croctalus, moccasin venom) and suggest that a lysis of lipoid constituents of the corpuscles is the fundamental change. They state that the lipolytic splitting of lecithin takes place under the action of many hæmolytic agents. This may explain the

¹ Bio-chem. Zeit. iv, p. 25, 1907; xiii, p. 363, 1908; Archiv für Hygiene, lxii, p. 339.

² Bio-chem. Zeit., v, p. 368, 1907; ix, p. 58, 1908; xiii, p. 234, 1908.

³ Berl. klin. Woch. ii, Jan., 1907.

⁴ Bio-chem. Zeit., iv, p. 281, 1907.

permeability of artificial lipid membranes for proferments observed by Swart⁵ or there may be adsorption, without splitting, of lipid.⁶

Arrhenius⁷ explains agglutination by acids as due to coagulation of proteins in the envelope.. The entrance of acids into the corpuscles and therefore their hæmolyzing power is accelerated by lecithin. But he gives no satisfactory explanation of how this is brought about.

Baumgarten⁸ restates his "osmological" explanation of hæmolysis. He finds the fundamental cause of the liberation of the blood-pigment in an altered permeability of the stroma. He correctly distinguishes between total destruction of the corpuscle and liberation of the hæmoglobin. Up to a certain point his ideas coincide with those expressed in my first paper ten years ago, and repeated and emphasized in several succeeding ones. In essential points also my long-published views do not seem to differ fundamentally from some of those more recently put forward by Koepe, Liebermann, and K. Meyer. Meyer⁹ examined the mechanism of saponin hæmolysis. He states that the first experiments on the mechanism of saponin-laking were undertaken by Ransom¹⁰ Two years earlier I published experiments¹¹ on this subject, in which by physico-chemical methods it was demonstrated that a change in the permeability of the corpuscles to electrolytes and water is an essential part of the saponin action. Later¹² it was shown that the action on the permeability of the corpuscles as regards ions still took place when they had been fixed by formaldehyde, and that the constituent on which the saponin acted might therefore be assumed to be non-protein. Ransom's valuable investigation concerns the constituent attacked. He says it is cholesterin. My experiments concern the immediate physico-chemical results of the attack and the way in which those physico-chemical changes lead to the liberation of blood-pigment. Meyer¹³ denies that Ransom's experiments prove that cholesterin is the point of attack. He concludes, himself, that the action of saponin on the lecithin of the corpuscles is the important thing.¹⁴

⁵ Bio-chem. Zeit., vi, p. 358, 1907.

⁶ Dauwe: Hofmeister's Beitr. vi, p. 426, 1905.

⁷ Bio-chem. Zeit., xi, p. 161, 1908.

⁸ Bio-chem. Zeit., xi, p. 21, 1908.

⁹ Hofmeister's Beitr. xi, p. 357, 1908.

¹⁰ Deutsch. med. Woch., xxvii, p. 194, 1901.

¹¹ Journ. of Physiol. xxiv, p. 224, 1899.

¹² Ibid., xxvi, p. 470, 1901.

¹³ Archiv für Hygiene, lxx, p. 293, 1908.

¹⁴ Cf. Pascucci: Hofmeister's Beitr. vi, p. 543, 1905, and Porges and Neubauer: Wien. klin. Woch., p. 1285, 1907; Bio-chem. Zeit., vii, p. 152, 1908.

Koeppel¹⁵ has attempted to explain the various kinds of hæmolysis as the result of simple chemical and physical changes in the envelope of the corpuscle. The appearance of novelty in these conclusions is partly obtained by his ignoring the results of previous workers. On the whole also his explanations are too "simple." For example, heat-laking is due, he says, really following Hermann,¹⁶ to the melting of the fatty substances in the wall of the corpuscle when the temperature corresponding to their melting point is reached. This does not explain why in cautious heat-laking the stromata still contain the greater part of their electrolytes, whereas, in saponin-laking the electrolytes leave the stromata in relatively large amounts. Nor does it explain why electrolytes can be brought out of the ghosts of heat-laked blood by saponin, a substance which is believed to act on the corpuscles in virtue of its power of dissolving lipoids, and to an equal extent by water, which must affect the corpuscles in a different way. Again, Koeppel's explanation of heat-laking takes no account of the fact that although ether and saponin greatly increase the conductivity of formaldehyde-fixed corpuscles by acting on the lipoids, heating formaldehyde corpuscles to the temperature of heat-laking does not increase the conductivity of the suspension, but may even slightly diminish it just as in normal blood.¹⁷ Further, the temperature at which heat-laking occurs is not a perfectly definite temperature, but depends both on the duration of the heating and on the condition of the corpuscles. The rapidity and completeness of heat-laking also depend on the nature and the concentration of the suspending liquid. For instance, cane sugar delays laking although it does not prevent it.¹⁸ These observations have been confirmed by Gros.¹⁹

Peskind²⁰ has carefully investigated the mechanism of ether-laking and sums up "in favor of the hypothesis that the cause of ether-laking is the solution and extraction of cholesterin and lecithin from the envelopes of the corpuscles." "Yet," he cautiously adds, "a more extended research may show that it is only the modification of these substances by ether and not their solution that is the cause." For he found that ether saturated with cholesterin caused laking in the same strength as ether itself.

¹⁵ Pflüger's Archiv, xcix, p. 33, 1903.

¹⁶ Pflüger's Archiv, lxxiv, p. 164, 1899; xci, p. 164, 1902.

¹⁷ Stewart: Journ. of Med. Research, viii, p. 307, 1902.

¹⁸ Ibid., p. 285.

¹⁹ Archiv für exp. Path. und Pharm., lvii, p. 64.

²⁰ Amer. Journ. of Physiology xii, p. 184, 1904.

Bang²¹ repeated Peskind's experiment with the same result, although he does not mention Peskind's work in his extensive article. Bang showed also that ether saturated with lecithin and cholesterin caused laking as rapidly as ordinary ether. He therefore asserts that neither lecithin nor cholesterin is the substance affected by ether in ether-laking. This reasoning is not conclusive. For some ether might be expected to be taken up by the surface of the corpuscles by adsorption²² or distributed over the lecithin and cholesterin of the envelopes in accordance with the partition-coefficient, some of the lecithin and cholesterin in the external ethereal solution being precipitated. Thus, as Peskind pointed out, even although no lecithin or cholesterin might be actually removed from the corpuscle, the condition of the envelope might be altered so that it became more permeable to water and electrolytes. Some ether might be expected also to penetrate to the interior of the corpuscle. Arrhenius²³ has shown how great the concentration of hæmolytic substances in the corpuscles may be in comparison with the surrounding liquid, saponin, for example, when equilibrium is established, having a concentration 120 times greater, and ammonia a concentration 880 times greater in the corpuscles than in the liquid. In this connection, the idea developed by J. Traube²⁴ of the relation between surface tension and hæmolysis is interesting. Starting from the theory of Willard Gibbs, that substances which diminish the surface tension of the solution strive to wander into the surface so that the concentration of such substances in the surface layer becomes greater than in the interior of the liquid, he shows that if we bring a watery phase together with solid phases, as blood cells or bacteria, or together with another liquid, for example, a lipoid solution, the substances which diminish the surface tension of the water most will collect in greatest

²¹ *Ergeb. der Physiol.* Jahrg., vi, p. 183, 1907.

²² The term "adsorption" is not always used in physiological writings in a perfectly definite sense. Freundlich considers adsorption to be a process of (physical) condensation of the adsorbed substance upon the surface of the adsorbent. (See paper by L. L. and D. D. Van Slyke: *Journal of Bio-chemistry*, iv, p. 259, 1908.) But many writers use the term also for processes not always understood and which seem to include certain chemical reactions. Perhaps "mechanical adsorption" might be advantageously employed (as by Michaelis and Rona: *Bio-chem. Zeit.*, xv, p. 196, 1908) to distinguish the physical process.

²³ *Bio-chem. Zeit.*, xi, p. 161, 1908.

²⁴ *Bio-chem. Zeit.*, x, p. 371, 1908; *Pflüger's Archiv*, cv, pp. 541, 559, 1904; Traube and Blumenthal: *Archiv für exp. Path. und Ther.*, xxii, p. 117, 1906; Traube and Bickel: *Deutsch. Med. Woch.*, p. 28, 1905.

concentration on the surface of the foreign phase, and therefore tend to be adsorbed as well as dissolved to the greatest extent in the foreign phase. Such substances as ordinary alcohols, esters, ethers, ketones, also substances like saponin and bile-acids must for this reason pass from physiological salt solution into the lipoid envelopes of the blood corpuscles.

Noguchi²⁵ has pointed out the similarity of the lipolytic form of hæmolysis to the ordinary amboceptor-complement hæmolysis.

Von Dungern and Coca²⁶ attribute the hæmolytic action of cobra venom solely to a lipolytic ferment, and, like Bang,²⁷ deny the existence of compounds between lecithin and cobra toxin (toxolecithids) such as those described by Kyes²⁸ who, however, has ably defended his position.²⁹

Dautwitz and Landsteiner³⁰ see in hæmolysis by sera and toxins, as the essential change, a rupture of the combination of protein and fat-like substances on which the integrity of the cell depends.³¹

I have long been convinced that the mechanism of hæmolysis can only be thoroughly understood when we understand the physico-chemical properties of the erythrocytes and the relation of these properties to their histological structure. A study of the erythrocytes in this regard, besides being of interest in itself, is calculated to throw light upon the relations of the electrolytes of the cells in general to the colloids and to that complex which we call protoplasm, relations which constitute one, or rather a group of the most important problems of physiology and pathology. There is every reason to believe that these relations are intimately concerned in the maintenance of the normal osmotic equilibrium of the cells and liquids and, therefore, in all physiological processes which depend upon the preservation, and in all pathological processes which depend upon the upsetting of that equilibrium. In particular, we may mention as of interest in this connection, such physiological processes as secretion, absorption, the contraction of muscle, the conduction of nerve impulses and, possibly,

²⁵ Bio-chem. Zeit., vi, p. 185, 1907.

²⁶ Ibid., xii, p. 407, 1908.

²⁷ Ibid., xi, p. 520, 1908.

²⁸ Berl. klin. Woch. Nos. 42 and 43, 1903.

²⁹ Ibid., iv, p. 99, 1907.

³⁰ Hofmeister's Beitr. ix, p. 431, 1907.

³¹ Cf. Landsteiner and Jagic: Wien. klin. Woch., 1904; Münch. Med. Woch., 1904.

fertilization and growth³² and such pathological processes as œdema and hæmolysis. How the electrolytes of the plasma of blood and lymph are related to the proteins of these liquids, how and to what extent they pass into the cells, in what physical or chemical conditions they exist in the intracellular contents, what consequences follow the increase or diminution of normal electrolytes or the introduction of abnormal ones, what the regulatory mechanisms are which provide against variations beyond the physiological limits, all these are questions of great interest. None of them, I venture to think, have been exhausted, despite the large amount of attention they have excited in recent years.

The blood and lymph corpuscles have this peculiarity among cells that their whole surface is in contact with a liquid of uniform chemical composition and uniform molecular concentration. Although this is not the case for the whole of the blood or lymph, we can certainly assume that it is so for the small portion surrounding a single corpuscle, moving freely in the blood stream. It may be far from true, however, for a corpuscle which clings for a time to the walls of a capillary through which an active exchange of material is going on. Such a corpuscle may be in some respects in the position of a corpuscle at the edge of a drop of blood on a slide, which may lake even before any visible drying occurs. Other cells, such as glandular epithelium, are very differently situated. The basal surface of a gland cell is in contact with lymph and the alveolar surface with the secretion of the gland. The epithelial cells of the intestine are in a similar position. Their free ends are in contact with the contents of the gut, their deep ends with lymph. Whether or not we consider the envelopes of cells as related to precipitation membranes³³ in which are concentrated certain constituents of the liquid phase with which they are in contact, or which they enclose, or as surfaces of separation between a solid and a liquid phase in which substances that diminish the surface tension collect,³⁴ there can be no doubt that their properties must depend to some extent

³² J. Loeb: Pflüger's Archiv, lxxxviii, p. 68, 1901; Amer. Journ. of Physiol. vi, p. 411, 1902; Bio-chem. Zeit., v, p. 351, 1907; etc. Cf. A. P. Mathews, Amer. Journ. of Physiol. xviii, p. 89, 1907.

³³ Zangger: Ergebnisse der Physiologie, Jahrg., vii, p. 98, 1908.

³⁴ Traube: Loc. cit.

on the liquids with which they are normally in contact. So that it is not permissible to speak of the permeability of, say, the epithelium of the convoluted tubules as a property constant, under given conditions and for a given substance, for the whole periphery of the cell. For the mammalian colored corpuscles, the boundary which separates the homogeneous intracorpuseular contents from the homogeneous plasma may be considered as possessing a uniform permeability throughout its whole extent. But the kinds of cells for which this assumption can be made are few. Even in the nucleated erythrocytes there are indications that absolute uniformity does not exist unless, perhaps, at points symmetrically placed with reference to the nucleus.

For example, in the action of various laking agents on the colored corpuscles of *Necturus*, it may be seen that the poles of the corpuscle farthest from the nucleus are first attacked. And when swelling of the nucleus is caused by treating sublimate-fixed corpuscles in certain ways, the corpuscles do not yield symmetrically to the swelling nucleus. In the leucocytes the differentiation of internal structure probably carries with it a still greater lack of uniformity in the permeability of the periphery at various points. The same seems to be true of striated muscle fibers, the permeability of which for certain ions appears to be greater at the end attached to the tendon than elsewhere.

The so-called irreciprocal permeability of frog's skin³⁵ and of intestinal mucosa³⁶ are probably illustrations of this. It is not absolutely necessary to assume that in the latter case the phenomenon depends on the presence of two histological layers in the membrane, as Hamburger has suggested. The two surfaces of the epithelial cells must of themselves constitute such a double membrane. Even a double membrane of parchment paper-collodion shows irreciprocal permeability, pepsin passing more easily in the direction from parchment to collodion, water more easily in the opposite direction.

I have already published a considerable number of papers on certain aspects of several of these questions, and promised³⁷ to discuss in detail in a future communication the *modus operandi* of the various

³⁵ Bayliss: *Bio-chem. Zeit.* xi, p. 226, 1908.

³⁶ Hamburger: *Ibid.*, p. 443, 1908.

³⁷ Amer. Journ. of Physiology, viii, p. 103, 1902.

hæmolytic agents investigated. I now desire to redeem this promise by bringing together, as far as possible, in a general view the deductions which seem to follow from my own observations, comparing and supplementing them with results obtained by others.

The investigation of certain physico-chemical properties of the corpuscles and their relation to the structure of these cells will constitute the first half of this paper, the second half being occupied more specifically with the mechanism of hæmolysis. But this division is merely for convenience in the treatment of what is after all a single theme, and matters which formally belong to one division may not infrequently be referred to in the other.

PART I. CERTAIN PHYSICO-CHEMICAL PROPERTIES OF ERYTHROCYTES AND THEIR RELATION TO THE HISTOLOGICAL STRUCTURE OF THESE CELLS

Historical Note.—The fundamental fact that the colored corpuscles are practically non-conductors was first observed by me in an investigation on the output of the heart, by a method involving the measurement of the conductivity of numerous samples of blood before and after the injection of sodium chloride solution into the left ventricle. The first communication was presented to the American Physiological Society in December, 1896.³⁸

A more particular account of the phenomenon was published in the Journal of the Boston Society of Medical Sciences No. 16, June 15, 1897. The paper was dated June 3. I there stated

1. That the electrical resistance of defibrinated blood is from two to five times greater than that of the serum.
2. That when defibrinated blood is centrifuged and samples taken from different portions of the tube, the resistance increases with the proportion of corpuscles in the sample. The resistance of a sample from the bottom of the tube may be fifteen times that of the serum or even more.
3. Since in such sediments there is always some serum between the corpuscles the conclusion seems warranted that in comparison with the serum the blood corpuscles are non-conductors.

Thus, a sediment with conductivity only $\frac{1}{16.5}$ of that of the serum still contained 12 per cent of serum by volume. Could this have been all

³⁸ Science, Jan. 22, 1897.

removed, the conductivity would have been enormously less. In another experiment³⁹ a sediment had the conductivity of 3.88 the serum 86.22, the defibrinated blood 29.48. That is, the conductivity of the sediment was only $\frac{1}{22}$ of that of the serum, although the sediment still contained 7.7 per cent of serum. Bugarsky and Tangl obtained sediments with a conductivity only $\frac{1}{30}$ of that of the serum, and these of course were still admixtures of serum and corpuscles.

In discussing the meaning of this remarkable fact, I pointed out that two explanations suggest themselves.

(a) That the corpuscles, while containing dissociated ions and therefore capable of electrolytic conduction in their interior, are surrounded by a non-conducting envelope—an envelope, let us say, which refuses passage to the ions that exist in the blood, but not necessarily an envelope structurally differentiated from the rest of the corpuscle.

(b) That throughout the whole substance of the corpuscle the bodies (inorganic salts) which would otherwise behave as electrolytes are combined with non-conducting, non-dissociable molecules, (hæmoglobin and other proteins,) and thus rendered for the time non-dissociable.

In attempting to decide between these two explanations, I quoted the results of experiments on laking the blood in various ways, some of which seemed consistent with either explanation, but others consistent only with the second. For example, when blood is rendered laky by repeated careful freezing and thawing, the resistance is not in general lessened, but may be somewhat increased; but if distilled water be now added to the already laked blood, the resistance (allowing for the dilution) is diminished just as if the water had been originally added to unlaked blood. This seemed to show that not only may the inorganic salts of the corpuscles be bound in non-dissociable combinations within the intact corpuscles, but that those combinations, or some of them, can exist even after the corpuscles have been laked so long as they are not exposed to the dissociating influence of dilution. It was pointed out that the stability of the quantitative and even qualitative differences in the easily diffusible inorganic constituents not only between the blood corpuscles and plasma, but between the organized material and the liquid of the tissues in general seems to require some such theory for its explanation.

About a month later W. Roth⁴⁰ announced that the conductivity of

³⁹ Journ. of Med. Research, viii, p. 305, 1902.

⁴⁰ Centralblatt für Physiologie, xi, July 10, 1897.

the red corpuscles was so small that they could be considered non-conductors. I thereupon called attention to my results,⁴¹ giving specimens of the numerical data and indicating how the phenomenon in question might be used to determine the relative volume of plasma and corpuscles without the more or less artificial changes entailed by other methods, such as the hæmatocyte method even when the admixture of salt solutions is avoided.

The statement of L. Löhner in a recent paper⁴² that Burgarsky and Tangl first showed that the red corpuscles are bad conductors is incorrect.⁴³

Pursuing the subject, I presented⁴⁴ certain facts which showed that the still relatively high resistance of blood laked by gentle means (careful freezing and thawing, careful heating to 60° C., foreign serum, spontaneous laking) was not due to the liberation of the electrolytes of the corpuscles in such a form that they could not conduct, since the addition of the more violent laking agents (water, supraminimal doses of saponin) to the sediment of the laked blood containing the ghosts caused a marked increase in the conductivity, while their addition to the serum of the laked blood caused a much smaller relative increase in conductivity, an increase similar to that obtained in normal serum.

Oker Blom, applying the same methods⁴⁵ but without reference to my work, published a paper containing tables and curves showing a striking similarity to mine. In subsequent work⁴⁶ he obtained further confirmation of my results, amplifying them in certain points.

Condition of the intracorpuselelectrolytes. These experiments, then, while they did not disprove the idea that the cause of the low conductivity of the corpuscles is the existence of the electrolytes in them in such a condition that they cannot conduct, lent it no support because they failed to discover evidence of the liberation of such compounds when the hæmoglobin escapes from the corpuscles in the act of laking. In short, the net result of the work up to this point as regards the question we are discussing, was to throw back the inquiry

⁴¹ Ibid., August 7, 1897.

⁴² Pflüger's Archiv, cxx, p. 193, 1907.

⁴³ Bugarsky's paper was published in the Centralblatt für Physiologie, July 24, 1897.

⁴⁴ Journ. of Physiol., xxiv, p. 211, 1899.

⁴⁵ Pflüger's Archiv, lxxix, p. 111, 1900.

⁴⁶ Ibid., lxxix, p. 510; lxxx, p. 167, 1900.

from the intact corpuscle to the shadow. This statement is based on such evidence as the following obtained with several methods of laking.

Spontaneous Laking. 1. The extracorpuseular liquid of dog's defibrinated blood which had been laked by standing eight days at room temperature, was filtered free from ghosts. It still contained the hæmoglobin in solution. The conductivity of the liquid, when corrected for the depressing influence of the hæmoglobin,⁴⁷ was of the same order of magnitude as that of dog's serum, while the conductivity of the laked blood was of the order of magnitude of that of dog's defibrinated blood, the relation between the two being 3.6 : 1. On diluting the filtrate with water, the relative increase of conductivity was only as much as occurs in the case of normal serum.

2. When two suspensions of corpuscles in serum, the one rich and other poor in corpuscles, undergo spontaneous laking under the same conditions, the conductivity of the second suspension, when laking is complete, is many times greater than that of the first. The conductivities are of the same order of magnitude as those of suspensions rich and poor respectively, in intact corpuscles. The addition of water or saponin causes a relatively great increase in the conductivity of sediments rich in ghosts and a relatively small increase in conductivity of suspensions poor in ghosts. The conclusion is, therefore, warranted that the ghosts in parting with their hæmoglobin have not in this form of laking parted with any large proportion of their electrolytes. They remain relatively bad conductors not because they have lost their electrolytes but because their relation to electrolytes, both their own and those of the serum, remains the same as that of the intact corpuscles notwithstanding the discharge of the hæmoglobin.

Heat-laking. With cautious heat-laking a similar result is obtained.

⁴⁷ Hæmoglobin, like other non-conductors, depresses the conductivity of a solution of electrolytes (in the case of serum, by 1.88 per cent for each gram of dog's oxyhæmoglobin dissolved in 100 cc. of serum.) I stated long ago (Studies from Physiol. Laboratory, Owen's College, Manchester, p. 144, 1890) that when oxyhæmoglobin is dissolved in distilled water there is no convection of it under the influence of a voltaic current. Pauli (Hofmeister's Beitr., vii, p. 531, 1906) has recently shown that the same is true for the serum proteins freed from electrolytes by dialysis. Like other colloids, oxyhæmoglobin exhibits electrical convection when it acquires a charge in solutions of electrolytes (Gamgee: Proc. Roy. Soc. lxx. p. 79, 1902.)

The extracorporeal liquid, freed from ghosts, has a conductivity of the same order of magnitude as that of the blood serum while the conductivity of the sediment is much less. Dilution with water produces a much greater relative increase in the conductivity of the sediment than of the liquid. Therefore, the influence of water in increasing the conductivity of heat-laked blood, is exerted mainly on the ghosts and not on the extracorporeal liquid. The increase of conductivity produced by saponin in heat-laked blood can in like manner be shown to be due to an action on the ghosts.

Saponin. The following facts show that the increase of conductivity produced by saponin (or sapotoxin), in the case of fresh blood, is not due to the breaking up of protein-salt compounds in the extracorporeal liquid:

1. Saponin does not produce such an effect on serum.
2. When saponin acts on defibrinated blood at 0° C. a stage can be found, when the dose of saponin is not too great, in which the conductivity is increasing while as yet no hæmoglobin has escaped from the corpuscles. The increase in conductivity, of course, cannot be due to decomposition of combinations of electrolytes in the serum. It is probably due to increased permeability of the corpuscles to ions, but possibly to some extent to enrichment of the serum with corpuscular electrolytes. As soon as the exit of hæmoglobin begins, the conductivity falls somewhat (depressing influence of the hæmoglobin as a non-conductor) and goes on falling until all the hæmoglobin is liberated, if the dose is no larger than suffices to cause its complete liberation. With a larger dose the first stage of increased conductivity is still seen and also the stage of decreased conductivity. But the latter does not go so far and is succeeded during the exit of the last portion of the hæmoglobin by an increase of conductivity (due to escape of electrolytes and diminution of the mechanical effect of the corpuscles on the conductivity). With still larger doses⁴⁸ the second stage of diminished conductivity may be absent or very slightly marked. Here the depressing influence of the hæmoglobin is overcome by the liberation of electrolytes from the start.

It is a remarkable circumstance that when the saponin acts at

⁴⁸ Experiment iv, Amer. Journ. of Physiol., ix, p. 87, 1903.

0° on washed corpuscles⁴⁹ suspended in saline instead of on defibrinated blood, the stage of increased conductivity is either absent or is so short that practically, with minimal doses of saponin, the conductivity goes on diminishing steadily so long as hæmoglobin is liberated. In experiment VI⁵⁰ with a large dose of saponin acting on washed corpuscles, no stage of diminished conductivity can be detected. The saponin causes a marked increase in conductivity, whereas in the same suspension with a small dose the stage of diminished conductivity is very distinct, not being preceded, however, by a stage of increased conductivity. The most probable explanation of these differences is that a serum constituent (in the case of defibrinated blood) which combines with or dissolves saponin (cholesterin?) hands it over slowly to the corpuscles, or is itself taken up by the envelope and continues there to protect the corpuscle, so that the first stage of increased permeability without liberation of the hæmoglobin is brought about. The envelope alone being affected, the combination between the blood-pigment and the stroma is not loosened at this stage. Or it may be that such effect as is produced on the envelope is only great enough to allow ions to pass through but not yet hæmoglobin. A similar phenomenon is seen in the case of bile salts acting at 0°, although the preliminary increase of conductivity is less marked.⁵¹

3. The conductivity of the extracorporeal liquid after laking with saponin is not increased, allowance being made for the depressing influence of hæmoglobin, but that of the sediment is greatly increased⁵²

4. The conductivity of the extracorporeal liquid after saponin has acted on formaldehyde-fixed corpuscles is such that we must conclude that the increase of conductivity of the suspension is due to an increase of conductivity in the corpuscles and not in the extracorporeal liquid.

That saponin produces its effect on the conductivity of blood in the same way whether the blood is fixed by formaldehyde or is fresh is indicated by the following facts:

1. When blood is gradually fixed by formaldehyde and acted on by

⁴⁹ Experiment vii, *Ibid.*, p. 91.

⁵⁰ *Ibid.*, p. 89.

⁵¹ *Ibid.*, p. 93.

⁵² *Journal of Experimental Medicine*, vi, p. 275, 1902.

saponin from time to time, the percentage increase of conductivity produced by a given dose of saponin is the same whether laking occurs or not.⁵³ This is especially true when such doses of saponin are used as suffice to produce complete laking in unfixed blood. When smaller doses are used, the depressing influence of the liberated hæmoglobin on the conductivity of the extracorporeal liquid may mask the increased conductivity of the partially laked corpuscles or the ghosts and cause the conductivity of the unfixed blood to undergo a smaller alteration under the influence of saponin than the fixed blood.

2. When the percentage increase in conductivity produced by a given dose of saponin on unfixed blood is compared with that produced on fixed blood, the effect is found to be approximately the same, provided that allowance is made for the depressing influence of the hæmoglobin.

How does saponin increase the conductivity of the corpuscles? There are three possibilities: (a) by increasing the permeability of the corpuscles to the ions of the suspending liquid; (b) by increasing the dissociation of ions inside the corpuscle; (c) by increasing the permeability of the corpuscles to intracorporeal ions. Granted that (a) takes place, the conductivity of the corpuscles may be greatly increased by (b) and (c). If (b) alone occurs without (a) or (c) no effect can be produced on the conductivity either of corpuscles or suspending liquid.

If (b) and (c) happen without (a), the conductivity of the suspending liquid and, therefore, the conductivity of the suspension would be increased by the enrichment of the liquid with intracorporeal electrolytes while the corpuscles remain non-conductors.

In the case of saponin acting on formaldehyde corpuscles (a) must be assumed to have taken place, since when a suspension is treated with saponin the conductivity of the sediment after centrifugalization is found to be increased, even when the conductivity of the suspending liquid is not increased or is diminished. And according to Woelfel's observations⁵⁴ when formol corpuscles suspended in cane sugar solution are acted upon by saponin, no increase in the ash of the sus-

⁵³ Journ. of Physiology, vol. xxvi, p. 480, 1891.

⁵⁴ Bio-chem. Journ., iii, p. 146, 1908.

pending liquid takes place. So we may assume that neither (b) nor (c) occurs; or that if (b) occurs, (c) does not. I have shown that saponin exerts its normal action on unfixed corpuscles in cane sugar solution.

Even in saturated cane sugar solution laking of unfixed corpuscles is produced by saponin as rapidly as normal. It is interesting to observe how suddenly the greatly crenated corpuscles swell up and assume a spherical form before the blood pigment escapes. There is every reason to believe that saponin exerts its normal action in the presence of cane sugar on formaldehyde corpuscles too. We are left, then, to the conclusion that saponin increases the conductivity of the formaldehyde corpuscles by increasing their permeability to the ions of the serum. We have already recognized an increase of permeability of the fresh corpuscles at 0° C. as the first stage in the action of saponin and have shown that this coincides with the taking up of saponin by the corpuscles.

When cholesterin and lecithin are extracted from formaldehyde corpuscles by ether, the conductivity of the corpuscles is markedly increased and now saponin produces no effect. The inference seems clear that the saponin affects the formaldehyde corpuscles by acting on the ether-soluble substances. This action, as has been said, must be of such a nature as to permit the ions of the suspending liquid to pass more easily through the corpuscles than before. It must, therefore, certainly affect the surface layer of the corpuscles whether it exerts any action in the interior or not. This agrees very well with the idea that lipoid substances form essential constituents of the surface layer of the corpuscles.

A further fact which indicates that the saponin acts on the surface layer of the formaldehyde-fixed corpuscles when it increases their conductivity is that, after the action of saponin, the corpuscles do not clump so readily as before and are more easily dispersed by shaking.

Recent work has confirmed the idea expressed by Peskind⁵⁵ that the envelope of the corpuscles is not to be considered as composed exclusively of lipoids, according to Overton's assumption,⁵⁶ but that protein, probably nucleo-protein, is also an important constituent.

⁵⁵ Amer. Journ. of Physiol. viii, p. 404, 1903.

⁵⁶ Studien Ueber die Narkose, Jena, 1901.

Höber⁵⁷ has brought forward evidence that neutral salts may alter the permeability of the superficial layer of the corpuscles by causing changes in the colloids of that layer. The series in which the ions can be arranged as regards intensity of action, under given conditions, correspond approximately to the series for precipitation of proteins (egg-albumin). But according to Porges and Neubauer,⁵⁸ who repeated the work of W. Koch,⁵⁹ although the order of the ions is the same as regards their effect on lecithin suspensions as the series found by Hofmeister and Pauli for albumin, the concentrations in which they act agree much better with the physiological limits in the case of lecithin. They therefore give to the lipoids the preponderant rôle in the envelope.

Since, in the formaldehyde corpuscles the hæmoglobin is certainly, and the other proteins probably, altered profoundly, while the corpuscles still remain practically impermeable to the ions of the serum, the suggestion is that the surface layer consists in preponderating amount of substances not essentially altered by formaldehyde.

Bile-salts. These substances can still be acted on by *bile-salts*. For sodium taurocholate causes an increase in the conductivity of formaldehyde corpuscles similar to that caused by saponin, and without any increase in the conductivity of the suspending liquid⁶⁰.

In the experiment cited, the conductivity of the control of the formaldehyde blood was 52.38, that of the formaldehyde blood after the action of the bile salt 74.78. The conductivity of the unfixed blood after laking by the taurocholate was 54.99, that of the control 59.37.

It is a point worthy of note that when unfixed corpuscles, either in natural suspension in serum or suspended in salt solution, are laked by sodium taurocholate no increase in the conductivity occurs, or rather a diminution due to the influence of liberated hæmoglobin.

Thus in one experiment⁶¹ the conductivity, at 0° C., of defibrinated blood when 28 per cent of the hæmoglobin had been liberated was 32.21. That of the control (to which sodium chloride solution had been

⁵⁷ Bio-chem. Zeit., xi, p. 35; xiv, p. 209, 1908.

⁵⁸ Bio-chem. Zeit., vii, p. 152, 1907.

⁵⁹ Zeit. für Physiol.-Chem., xxxvii, p. 181, 1903.

⁶⁰ Journ. of Med. Research, loc. cit., experiment 2.

⁶¹ Experiment 9, Amer. Journ. of Physiol., ix, loc. cit.

added so as to make up approximately for the direct increase of the conductivity of the blood caused by the addition of the sodium taurocholate solution) was 38.06. The amount of serum in the defibrinated blood was 58 per cent (by the electrical method). Taking the total hæmoglobin of the blood as 13 per cent this would give an amount of hæmoglobin in solution in the serum equal to 6.4 gr. per hundred cc. of serum. This of itself would reduce the conductivity by 11.5 per cent. Deducting this from the conductivity of the control, we get 33.7. So that at this time no sensible amount of intracorpuseular electrolytes would seem to be taking part in the conduction. After complete laking the conductivity was 33.22. If we suppose the hæmoglobin now to be uniformly distributed over the laked blood, the conductivity of the control, after making the requisite deduction for the depressing effect of the hæmoglobin in the laked blood, would be 29.1. This is certainly too low since the whole of the hæmoglobin in laked dog's blood cannot be held in solution. So that even now if intra-corpuseular electrolytes have been liberated the amount must be comparatively small. The effect on the ghosts is obviously quite different from that of saponin or water.

The explanation probably is that the bile-salt in general causes laking without entrance of water into the corpuscle, at least in moderate doses. The hæmoglobin is therefore separated from the stroma and discharged through the envelope without dissociation of stroma electrolytes to any great degree.

Bile-salt in a certain concentration seems to dissolve the envelope so rapidly that swelling of the corpuscle cannot take place, no difference of osmotic pressure being established when the envelope has been extensively injured, or when the bile-salt destroys or lessens the affinity of the corpuscle for water. It is quite in harmony with this view that when laking by taurocholate is caused to proceed very slowly some of the erythrocytes may be seen to swell up before the hæmoglobin escapes. In this connection the fact that under the influence of taurocholate, the leucocytes in *Necturus* blood swell little if at all, although they may become dim, is of interest. In water they swell greatly, as do the nuclei, the contours of cell and nucleus becoming very sharp. Here again, it is difficult to avoid the conclusion that the preservation of a relatively intact envelope is an essential condition for the entrance of a large amount of water.

Permeability of corpuscles for ammonium chloride. I endeavored to obtain evidence as to the possible condition of electrolytes in the interior of the corpuscles by investigating the absorption of ammonium chloride by unfixed and formol-fixed corpuscles. When ammonium chloride in substance is added to blood the corpuscles are not laked, although they take up the ammonium chloride freely. Notwithstanding the entrance of a relatively large quantity of ammonium chloride into the corpuscles and the presence of abundance of the ions of that salt in the serum, the corpuscles remain bad conductors like those of the original blood. Either, then, the ammonium chloride in the interior of the corpuscles is in such a condition that it does not ionize and is bound as rapidly as it enters, or the corpuscles after the affinities of the stroma have been satisfied and equilibrium has been established no longer admit any appreciable amount of ammonium chloride or, of course, of the normal serum electrolytes, although what has already entered or a portion of it may be ionized. When water is added to a sediment of corpuscles artificially enriched with ammonium chloride, electrolytes pass out of the corpuscles just as they do in the case of a sediment of normal blood, but to a much larger extent. The parallelism of the two curves shows that the ammonium chloride is escaping from the corpuscles in the same way as the normal electrolytes of the corpuscles do. We infer from this that the condition of the ammonium chloride in the interior of the corpuscles is not very different from that of the normal intracorpuseular electrolytes. Here, then, we have an instance of an electrolyte which readily penetrates the corpuscles, but whose ions, when equilibrium has been established, practically do not conduct the current from the serum across the corpuscles. The addition of water increases the conductivity of a suspension of corpuscles artificially enriched by ammonium chloride to a much greater degree than the conductivity of a suspension equally rich in normal corpuscles. This additional increase of conductivity may be due to two things; first, the liberation of ammonium chloride from the corpuscles and its solution in the serum; second, the conduction of electricity by ammonium chloride ions passing into the corpuscles from the serum when the envelopes or the corpuscles as a whole have been altered by the water. There is no question as to the first factor. That the second may have some influence is indicated by experiments

where the conductivity of the sediment of a mixture of formaldehyde-fixed corpuscles with ammonium chloride was found to be higher than that of the corresponding mixture of formol corpuscles with sodium chloride, while the conductivities of the serum of the ammonium chloride mixture and of the mixture itself were distinctly less than the corresponding conductivities for the sodium chloride mixture.

Mechanical laking. The results obtained on corpuscles laked by mechanical means (trituration with sand) are of special interest. One would suppose that this would be the most likely of all methods to permit the escape of the (hypothetical) electrolyte-colloid compounds without change. Fresh ox or sheep's blood was rubbed up in a mortar with silver sand previously incinerated and then washed thoroughly with distilled water and dried. Evaporation was prevented by covering the mortar with the copper rings of a water bath and conducting the trituration in a cool room. The rubbing was continued until the laking was practically complete, as controlled by microscopic examination and by centrifuging a small sample from time to time. Serum was separated from the defibrinated blood and hæmoglobin-containing liquid from the laked blood, evaporation being prevented by covering the centrifuge tubes with rubber caps. Freezing point and conductivity measurements were made on the original blood, on the laked blood after decantation from the sand without centrifugalization, on the serum and on the hæmoglobin-containing liquid after centrifugalization. In the experiment the results of which are quoted in Table I ox blood was triturated for twenty-five minutes. Ninety-five per cent. of the hæmoglobin was found to be in solution, representing an oxygen capacity of 22 cc. per hundred cc. of blood by Haldane's hæmoglobinometer. Besides the measurements of freezing point and conductivity a series of observations was made on the influence of dilution with water and of saponin on the defibrinated blood, the laked blood and the serum with the object of determining whether the laked blood follows the curve of a suspension of corpuscles like the defibrinated blood or of a homogeneous solution like the serum. As is seen from Table II and Fig. I, the curve for the diluted laked blood resembles that of the defibrinated blood much more than that of the serum. The same is true for the effect of saponin, which causes a marked increase in the conductivity of the laked blood and to exactly the same point as in

TABLE I.

	d	$\lambda (5^\circ) \times 10^8$
Defib. ox-blood.....	.530 } .531 } .531 }	39.06 } 39.18 }
Serum from the defib. blood.....	.531 .531 .532 .533 }	39.73* 80.22
Laked blood.....	.518 } .518 } .516 }	39.86 42.04† 42.11†
Laked blood (top).....		43.78‡ 43.93§

* After standing all night in ice-chest.

† After complete sedimentation of the sand. All night in ice-chest.

‡ After centrifuging one-half hour.

§ After centrifuging another hour.

TABLE II.

	DEFIB. OX- BLOOD.	LAKED BLOOD.*	SERUM.
+ 1 vol. water.....	39.67	43.19	77.76
+ 3 vols. water.....	24.28	30.69	45.11
+ 5 vols. water.....	17.48	18.99	24.14
+ saponin †.....	10.42	10.76	13.06
+ saponin †.....	54.52	54.52	73.48

* After separation of all the unlaked corpuscles, which made up not more than 0.3 cc. in 15 cc. of the laked blood. The blood was laked in the same way as in the experiment of Table I.

† One-tenth of its volume of an aqueous solution of saponin was added to the blood, etc. The saponin solution had a conductivity only slightly greater than that of the distilled water.

the defibrinated blood, while in the case of serum there is a small diminution of conductivity. The explanation is afforded by the microscopic observation that the laked blood was crowded with ghosts. The action of water and of saponin is obviously, in the main at any rate,

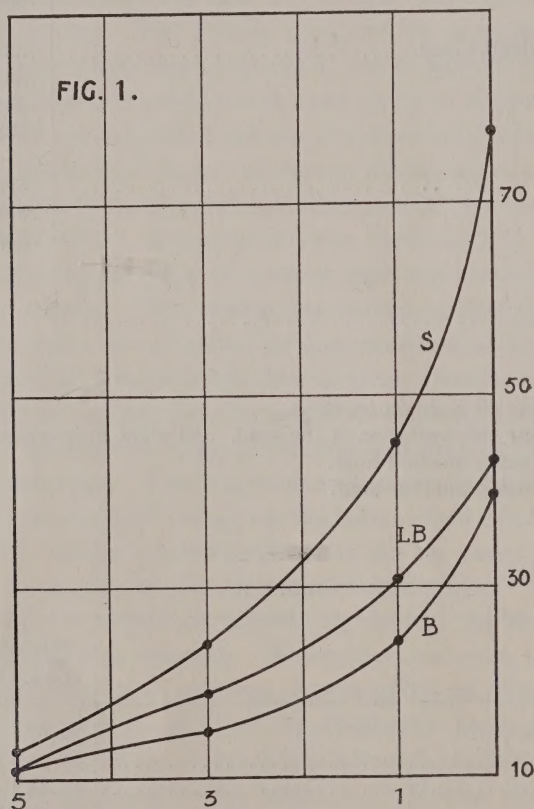


FIG. 1. Conductivities plotted along vertical and dilutions along horizontal axis. *S*, serum; *LB*, blood laked by trituration with sand; *B*, defibrinated blood.

an action on the ghosts and not on electrolyte-protein compounds liberated by trituration from the corpuscles.

In Table III, the results of an experiment in which sheep's blood was partially laked by shaking with clean mercury, according to the

method of Meltzer and Welch,⁶² are shown. It will be observed that in both methods of laking the λ of the defibrinated blood is somewhat diminished by mechanical laking. This agrees with the observation of Hamburger⁶³ and Foa⁶⁴ on the freezing-point of blood laked by freez-

TABLE III.

	λ	$\lambda(5^\circ) \times 10^8$
Serum from defib. sheep's blood.	.590 .593	89.90
The blood partially laked by shaking with mercury (top)	.582 .578 .586 .584	70.22
After further centrifuging (top)		76.13 74.78
The laked blood*		48.19 50.10 51.21
Another specimen of the laked blood.		
The laked blood + saponin (in substance)†		69.64
The defib. blood	.598 .590 .593 .588	48.38

* Forty-two per cent of the blood pigment was in solution, representing an oxygen capacity of 7.8 cc. per 100 cc. of blood, as determined by Haldane's hæmoglobi-nometer.

† The saponin causes only a very slight change in the conductivity of distilled water.

ing and thawing, and is in accordance with the conclusion of Moore and Roaf⁶⁵ that the osmotic pressure of the contents of the erythrocytes is normally somewhat less than that of the plasma.

⁶² Journ. of Physiology, v, p. 255, 1884.

⁶³ Archiv für Anat. u. Physiologie, p. 486, 1897.

⁶⁴ Archivio di Fisiologia, i, pp. 199, 342.

⁶⁵ Bio-chem. Journ., iii, p. 55, 1907.

Osmotic pressure of erythrocytes and of plasma. The conception of Moore and Roaf, that while there is equilibrium of osmotic pressure between the corpuscles and the plasma there is not equality, is not open to any *a priori* objection. Yet the method of proving this by direct freezing-point determinations on sediments rich in corpuscles is beset with difficulties. Höber⁶⁶ has well stated the objections to such measurements, not only in the case of blood corpuscles but also in the case of solid tissues. Doubtless, it is these difficulties which account for the fact that "in the whole extensive literature of cryoscopic measurements on blood" Moore and Roaf "have only been able to find one instance in which the freezing point of the separated corpuscles has been taken alongside that of the serum." It is obviously impossible to have that uniform distribution of fine ice-crystals in a mass like a blood-corpuscle sediment which is attainable in a liquid like serum. At best, we can stir up more or less completely frozen corpuscles. Further, we are not determining the freezing point of a homogeneous solution. Between the corpuscles are thin layers of serum in capillary spaces, in the interior of the corpuscles perhaps pockets of intracellular liquid in the interstices of the stroma, and I know of no means of assuring one's self whether or not the intracorpuseular and extracorpuseular liquids freeze at the same time, or of determining how much each contributes to the final result. The fact that the Δ of defibrinated blood is the same as that of its serum indicates that in a suspension containing abundance of serum the serum freezes at any rate as soon as the corpuscles. But in a sediment of corpuscles it might not be the same. If, now, while the real osmotic concentration were the same in the corpuscles and serum, and the intracorpuseular contents were to freeze before the serum because the latter is in much smaller capillary spaces, the first formation of ice in the corpuscles would cause an increase in their osmotic pressure by withdrawing water. Water might, therefore, pass into the corpuscles from the serum and so reduce the intracorpuseular concentration as to give too small a Δ for the corpuscles.

The influence of suspended particles on the freezing point of solutions, which has been studied by Tezner and Roska⁶⁷ may also play

⁶⁶ *Physikalische Chemie der Zelle u. Gewebe* p. 41.

⁶⁷ *Zeit. für Physiol. Chem.*, lvi, p. 495, 1908; and Tezner, *ibid.*, liv, p. 95, 1907.

a part. They showed that by adsorption dissolved substances accumulate at the surface of the suspended particles, leading to impoverishment of the suspending liquid and diminution of its Δ as compared with the Δ of the pure solution. In sediments rich in corpuscles the molecular concentration of the liquid between the corpuscles might thus be diminished, and in freezing point observations on the sediment the observed Δ might be smaller than that of the pure suspending liquid, although freezing of the intracorpuseular contents might not have occurred at all.

Cause of the low conductivity of the erythrocytes. To return to our original question as to the cause of the low conductivity of the corpuscles, the following statements are now permitted:

1. It must in part be due to a low degree of permeability for the ions of the plasma because the conductivity of the defibrinated blood is so much less than the conductivity of a dilution of the serum of the blood with a quantity of water equal in volume to the corpuscles, even when we allow for the relative increase of conductivity of the serum produced by dilution. We must assume that the corpuscles neither furnish ions to aid in the conduction nor permit the serum ions to pass as easily through them as through water.

2. It may be due entirely to the relatively low permeability of the corpuscles for the serum ions and intracorpuseular ions. Obviously, no matter how numerous the ions in the interior of the corpuscles might be, the corpuscles would still be non-conductors if the ions could not pass through them. A vesicle of fat enclosing a solution of electrolytes would conduct as badly as if the globule consisted entirely of fat.

H. Koeppe's⁶⁸ idea that we can assume with certainty that in the blood corpuscles all the salts are present in the form of neutral molecules and none dissociated since the corpuscles do not conduct, is erroneous, as I have pointed out already.⁶⁹

3. The low conductivity of the corpuscles may be due in part to the existence in them of electrolytes in the form of compounds with proteins or other colloids or in the form of adsorptates which render them for the time non-dissociable.

⁶⁸ Archiv für Anat. u. Physiologie, p. 504, 1899.

⁶⁹ American Year-book of Medicine, p. 501, 1900.

The envelope of the erythrocytes. In discussing the results of experiments with saponin on formaldehyde-fixed corpuscles several circumstances were indicated which point to this being an action on the surface of the corpuscles rather than on their interior, an action which permits ions to pass more easily through them so that a great part of the resistance which they normally interpose to the conduction of the current is abolished. The most probable analogy to this is the withdrawal or the modification of a membrane which interposes a resistance to the passage of ions in an electrolytic circuit.

This leads naturally to the question of the *existence of an envelope* in the erythrocytes. There has been much discussion as to this, especially in the case of mammalian corpuscles.

Waldeyer⁷⁰ sums up his position in these words: "Of the existence of a rigid (feste) cortical layer of the red corpuscles I am completely convinced from my own observation. But the differentiation has not yet progressed to the stage of a true membrane capable of isolation." von Ebner⁷¹ says: "One must assume that the stroma has a denser surface layer, which is insoluble in water and capable of osmotic phenomena similar to the ektoplasma of a naked living protoplast. Such a structure, which, according to Schulze, is described as a crusta, cannot be called a membrane."

Deetjen⁷² concluded that an outer envelope of extreme delicacy exists in mammalian corpuscles and claims to have demonstrated it histologically.

In a paper already cited⁷³ I described the appearances of the ghosts of mammalian corpuscles laked in various ways, especially after partial fixation, and stained. The appearances indicated the existence of a stroma, in Rollett's sense, bounded by a structure which in ruptured ghosts appeared like a ring with a double contour.

Peskind,⁷⁴ studying corpuscles in which the formation of bubbles of nitrogen had been caused by hydroxylamine hydrochlorate, showed the presence of an envelope, apparently free from blood pigment, which prevented the bubbles from escaping.

⁷⁰ Deutsch. med. Woch. Jahrg. xxi, p. 703, 1895.

⁷¹ Kölliker's Handbuch der Gewebelehre, iii, p. 741, 1902.

⁷² Archiv für path. Anat. u. Physiol., clxv, p. 283, 1901.

⁷³ Journ. of Physiology, xxvi, p. 494, 1901.

⁷⁴ Amer. Journ. of Physiology, viii, loc. cit.

Koepe,⁷⁵ staining the shadows with methyl violet, also convinced himself that envelopes exist in mammalian corpuscles.

Löhner⁷⁶ thinks there is an ectoplasmic layer, though not a separable membrane, in the mammalian corpuscles.

In the case of amphibian corpuscles there is good evidence of the existence of a distinctly differentiated envelope. W. Preyer⁷⁷ observed, in frogs and salamanders, erythrocytes in the process of division whose membrane was visible as a fine line with double contour stretching between the two portions of the corpuscle. F. Meves,⁷⁸ however, denies the existence of a membrane in amphibian corpuscles.

In the large colored corpuscles of *Necturus* I have demonstrated the existence of an envelope which can be histologically differentiated. It stains with Löffler's methylene blue after fixation of the corpuscles. When the corpuscles are ruptured in various ways the envelope may be separated from them as a histologically distinct structure. When intraglobular crystallization is caused in these corpuscles this structure is seen to be apparently hæmoglobin-free. This is of interest in connection with the fact already mentioned, that formaldehyde, which exerts a marked action on the blood-pigment, leaves certain of the normal properties of the envelope relatively unchanged. It may sometimes be seen that under the influence of sodium taurocholate, the envelope, after becoming distinct in consequence of the formation of hæmoglobin crystals inside the corpuscles, may disappear as if eaten away or dissolved. I have elsewhere⁷⁹ developed in detail the evidence for the existence of an envelope in the *Necturus* corpuscles. I will only add here, as further corroboration, the fact that the nuclear membrane is so distinct in these corpuscles and acts in such a way under the influence of reagents that it is not possible to doubt its existence as a histologically differentiated envelope. Now, the same staining procedure which brings out the nuclear membrane brings out the envelope of the corpuscle. Swelling and shrinking of the nucleus are not necessarily produced by the

⁷⁵ Pfüger's Archiv, cvii, p. 86, 1905.

⁷⁶ Archiv für mikros. Anat., lxxi, p. 129, 1907.

⁷⁷ Archiv für path. Anat. und Physiol., xxx, 1864.

⁷⁸ Anat. Anzeig., xxvi, p. 529, 1905.

⁷⁹ Amer. Journ. of Physiology, viii, loc. cit.

same reagents as cause swelling and shrinking of the corpuscle. It is quite easy to cause the corpuscle to swell while the nucleus remains unchanged in size, as by the action of sapotoxin, and, contrariwise, to cause swelling of the nucleus without alteration in volume or shape of the corpuscle, as by the action of sodium taurocholate. If the entrance of water into the nuclei or its exit is affected at all by the properties of the nuclear membrane, as it certainly must be, it is difficult to resist the conclusion that the passage of water into and out of the corpuscle is also influenced by the corpuscular envelope. And, indeed, it could be observed that after treatment of sublimate-fixed corpuscles with ammonium sulphide, the addition of Löffler's methylene blue caused the swollen corpuscles and nuclei to shrink to their normal size (osmotic action of the alcohol in the stain). This shrinking does not take place if the corpuscular envelope has been ruptured or so altered that it is no longer brought out by the methylene blue, even though the rest of the corpuscle appears normal. The inference is that the shrinking depends upon a property of the envelope rather than on a property of the interior stroma. For alcohol causes the passage of water out of corpuscles whose envelope is intact and therefore the osmotic pressure in the corpuscle rises, leading in turn to the passage of water out of the nucleus. If the envelope of the corpuscle has been ruptured no difference in osmotic pressure can exist between the interior and exterior of the corpuscle, and therefore the nucleus remains exposed to a hypotonic solution.

Another argument in favor of the view that the action of the formaldehyde on the superficial layer of the corpuscles is not so subversive of its normal properties as its action on the hæmoglobin and the constituents of the corpuscles more immediately related to the hæmoglobin is that corpuscles fully fixed by formaldehyde are capable of being agglutinated, or, at least, agglomerated, by specific sera.⁸⁰

Now, agglutination certainly involves some alteration in the surface layer of the corpuscles or in the relation of that layer to the agglutinating serum. The same suggestion is strengthened by another fact observed by me,⁸¹ that corpuscles fully fixed by formaldehyde cause,

⁸⁰ Cf. Noguchi: *Med. Bull. Univ. of Pennsylvania* xv, p. 327, 1902; Stewart: *Amer. Journ. of Physiol.* xi, p. 250, 1904.

⁸¹ *Amer. Journ. of Physiol.* xi, p. 250, 1904; xii, p. 363, 1904.

on injection into animals, the production of specific agglutinins and to a smaller extent of specific hæmolysins.

Now, whether the substances in the corpuscle which take up or fix specific agglutinin or specific hæmolysin are or are not identical respectively with the corpuscular constituents (agglutininogens, hæmolysinogens) which on injection of the corpuscles into animals of a different species give rise to the production of specific agglutinin or hæmolysin, there can be no doubt that there is a close relation between them. Some writers, like Liebermann⁸² and most of Ehrlich's pupils, maintain the doctrine of strict identity. Bang and Forssman⁸³ and Forssman⁸⁴ deny this. From its solubilities the active substance appears to belong to the somewhat loosely defined group of lipoids, although, as Takaki⁸⁵ points out, this is not absolutely proved. Coca⁸⁶ working with osmic acid-fixed corpuscles, obtained some results of interest in connection with my work on formaldehyde corpuscles. He finds that the specific agglutinins of rabbit's serum are quantitatively taken up by ox corpuscles fixed with osmic acid. Strongly fixed osmic acid corpuscles are still capable of binding specific hæmolysins, although they do not give rise on injection to their production. Lightly fixed corpuscles cause a slight production of amboceptor. He concludes that the capacity of protoplasmic constituents to bind specific antibodies is not always sufficient to give them the properties of antigens. But we need not assume with Bang and Forssman the complete independence of the production of antibody from the antibody-binding substance. Foreign serum seems to increase the conductivity of formaldehyde corpuscles distinctly,⁸⁷ although only slightly in comparison with saponin. The calculated conductivity is less than the actual by a small amount corresponding, perhaps, in the case of the heated serum, to the increase of conductivity produced by increasing the relative volume of suspending liquid to corpuscles. The suspending liquid separated by the centrifuge has practically the same conductivity whether heated or un-

⁸² Bio-chem. Zeit., xi, p. 405, 1908.

⁸³ Hofmeister's Beitr., viii, p. 238, 1906.

⁸⁴ Bio-chem. Zeit., ix, p. 330, 1908.

⁸⁵ Hofmeister's Beitr., xi, p. 274, 1908.

⁸⁶ Bio.-chem. Zeit., xiv, p. 125, 1908.

⁸⁷ Journ. of Med. Research, viii, p. 306, 1902.

heated serum is added. But the excess of conductivity for the suspension with unheated serum over the calculated conductivity is distinctly greater than for the heated serum suspension. The sediment of the suspension has a higher conductivity in the case of unheated than of heated serum, indicating that the permeability of the corpuscles has been somewhat increased by the unheated serum, the same change as occurs with saponin, only less marked. This is in favor of the view that the amboceptor-fixing substance of the corpuscle is not so altered by formaldehyde that it cannot act.

There is evidence that in the case of unfixed corpuscles the amboceptor alone can produce a certain change of permeability (influence of heated serum) (p. 117). The change of permeability in the formaldehyde-fixed blood is produced only by the action of the unheated serum. Yet, agglutination of formaldehyde-fixed corpuscles is caused by heated serum. Therefore, agglutination alone is not necessarily associated with increased permeability of the corpuscles to serum ions. This is an additional argument in favor of the conclusion that the agglutination and hæmolysis produced by foreign serum are not brought about by the same substance.

The precipitation of corpuscles by dilute acids and acid salts, without laking and without affecting the laking of the corpuscles after the removal of the precipitants, which has been studied by Peskind,⁸⁸ indicates that the action is on the peripheral layer of the corpuscles and that this layer is free from hæmoglobin. Peskind says that "the hæmoglobin contained in the corpuscles is not altered in the least if only the exact amount of reagent necessary to agglutinate and precipitate the corpuscles is employed. Yet all the precipitating agents in dilute solution produce alterations in solutions of the blood pigment. If moderate excess of reagent be added, the corpuscles will still be precipitated, but the blood spectrum shows no band in the red for five or ten minutes, depending on the amount of reagent employed. The same amount of reagent added to the same amount of saponin- or water-laked blood instantly changes part of the oxyhæmoglobin, and this conversion proceeds very rapidly to completion."

The recent work of Bohr⁸⁹ and others has indicated that the hæmo-

⁸⁸ Amer. Journ. of Physiology, viii, loc. cit.

⁸⁹ See Bohr's article in Nagel's *Handbuch der Physiologie*, i, p. 54, 1905.

globin in the corpuscles does not take up oxygen according to precisely the same curve as hæmoglobin in aqueous solution. This suggests that the absorption of oxygen by the corpuscles may be divided into two stages: (1) the passage of oxygen into the corpuscle through the envelope; (2) its combination with the hæmoglobin. Hüfner⁹⁰ also has remarked that a comparison of the manner in which oxyhæmoglobin in solution and blood pigment *in situ* in the corpuscles behave in the presence of reducing agents strongly suggests that in the latter case the pigment is protected from direct contact with the reducing substance in the plasma by the envelopes of the corpuscles. It would seem probable that if any portion of the original protoplasm of the colored corpuscle should be retained approximately unaltered in order to regulate the exchanges of the corpuscle and, therefore, to govern its nutrition as well as its function, it would be the peripheral portion which is in contact with the plasma. It would probably be advantageous for this that the protoplasm should not be loaded with a secondary product like hæmoglobin.

My view, then, is, that the low conductivity of the colored corpuscles, the leucocytes, and, presumably, of other cells is due (1) to the low permeability of the superficial layer of the cell for the ions of the plasma or lymph; (2) to a combination (or adsorption) of electrolytes with the proteins or other constituents of the corpuscle after their entrance into it. No evidence has been produced that all the electrolytes in the interior of the corpuscle are in such condition as to be incapable of conducting. Good evidence has been obtained that a portion of the intracorpuseular electrolytes is more easily liberated than the rest during hæmolysis. This portion may be in solution while the rest is adsorbed or combined.

Conditions governing the permeability of the erythrocytes. As to the ultimate reason of the relative impermeability of the corpuscles for the ions of the plasma, I have forborne to speculate.

It is certainly not an absolute impermeability. As Hamburger has shown,⁹¹ it is the relative impermeability for the kations only which normally prevents the passage of salts into the corpuscles, the

⁹⁰ Archiv für Anat. u. Physiologie, p. 463, 1907.

⁹¹ Ibid., p. 492, 1902.

anions being unable to enter without a corresponding number of kations. Under the influence of carbon dioxide, anions are able to pass into and out of the corpuscles. K. Spiro and L. J. Henderson⁹² have recently shown that with the aid of various substances which constitute an "inactive alkali reserve," the well-known phenomenon of the increase in the titratable alkalinity of the blood plasma under the influence of carbon dioxide can be imitated in artificial heterogeneous systems. The simplest assumption which will harmonize with the fact of the very low conductivity of the corpuscles is that the ions of the plasma and also the ions of the corpuscles, if such exist, are unable to pass except with difficulty through the bounding surface of the corpuscles. This assumption does not involve any theory of the property of the surface on which this relative impermeability depends. It can be made equally whether the impermeability is due to (a) difficulty of diffusion of the ions into or out of the corpuscle at its surface; (b) difficulty of solution of the ions in the substance of the corpuscle at its surface; (c) the relatively small adsorption of the electrolytes on the surface of the corpuscle. (d) If we do not include under adsorption, as is done by some writers, loose chemical combinations of ions with proteins or other constituents of the corpuscle, the relative impermeability may be due to the slight affinity of the outer portion of the corpuscle for these.

Any one of the possibilities mentioned may be true whether the corpuscle is homogeneous throughout or whether its outer portion is physiologically differentiated into a "Plasmahaut" or envelope, or histologically differentiated as well into an actual membrane. The existence, or the demonstrable existence, of such an envelope or of such a membrane is a question for separate consideration. The question whether, as a matter of fact, the corpuscles are relatively impermeable to certain substances, relatively permeable to others, can be investigated as such without reference to the question of the existence of an envelope.

That the serum ions in general do not easily penetrate the corpuscles may be regarded as proved, not only as a deduction from the low conductivity of the corpuscles suspended in serum, but by direct experi-

⁹² Bio-chem. Zeit., xv, p. 114, 1908.

ments.⁹³ I have myself shown, as was done later by Oker Blom, that the conductivity of isotonic sodium chloride solutions in contact with blood corpuscles remains practically unaltered. Therefore the ions of sodium chloride, quantitatively the most important ions of serum, do not easily penetrate the corpuscles even when their concentration in the serum is markedly increased. Whether this is due to low adsorptive power of the corpuscles to these ions, as Moore and Roaf suggest, really does not bear on our present question. The existence of potassium in the corpuscles, although they are also practically non-conductors when suspended in solutions of potassium salts, could only seem to be a difficulty, (a) if a special meaning were given to the term permeability, connecting it strictly with physical diffusion, for example; (b) if the impermeability were supposed to be absolute, which is not necessary to explain the low conductivity of the corpuscles; (c) if the permeability were supposed to be always the same for the undissociated molecules of a salt and for its ions; (d) if the corpuscle were assumed to preserve exactly the same degree of permeability to one and the same substance not only throughout its whole life history, with uniform external conditions, but also in different physiological states of the organs through which the blood passes or of the plasma in which the corpuscles float, and in the presence of varying concentrations of the substance in the plasma. There is no need to assume that the permeability of the developing erythrocytes is the same quantitatively and qualitatively as that of the adult cell. Nor is it our conception that the permeability is necessarily the same from hour to hour in the case of the fully formed corpuscles.

I have never asserted that either the selective permeability of the corpuscles for various substances or their reaction to various hæmolytic agents depends entirely on the properties of the surface layer. On the contrary, I have insisted on the part played by the stroma as a whole and have pointed out that the conception of the corpuscle as a vesicle, a little bladder filled with hæmoglobin solution, will not fit in with the facts of hæmolysis. I have not suggested, and do not imagine, that the permeability of the corpuscles, either for salts or for colloids, depends merely on diffusion through physical pores.

⁹³ Hedin, Eykman, etc.

Neither Traube's "atom-sieve" nor Ostwald's "ion-sieve"⁹⁴ is a very satisfying conception in the case of an animal cell, although the results of Voigtländer,⁹⁵ recently confirmed by K. Meyer,⁹⁶ show that in gelatin-gels the velocity of diffusion of salts diminishes as the concentration of the gels increases. In membranes poor in water like the shell membrane of the egg, the envelope of fat cells, the sarcolemma of muscle fibers, the neurilemma and medullary sheath of nerves, and in all probability (considering the low water content of the erythrocyte as a whole), the envelope of the colored blood corpuscles, the difficulty of diffusion of the ions may be itself a very important factor in the low conductivity.

It is not possible directly to test the question on ordinary cells whether inside the cells all the electrolytes are so bound that they do not conduct the current. In a large cell like a hen's egg, however, the question can be tested. Even if we hesitate to apply the results obtained on the albuminous coating, which conducts practically as well as an aqueous solution of the salts (apart from the ordinary depressing influence of the proteins on the conductivity) there can be no fundamental objection, so far as I can see, to applying the results obtained with the egg-yolk to the question whether ordinary cell contents conduct at all. Now, I have shown⁹⁷ that while the conductivity of the yolk is three times less than that of egg-white, it is still about one-fifth the conductivity of hen's blood serum. Whether this relatively low conductivity is due to a low degree of ionization of the salts or to mechanical causes (insulation by the fatty materials) I have not been able to show. The fact that dilution with water causes a much smaller proportional diminution in the conductivity of the yolk than in the conductivity of the egg-white might be explained as due either to the production of greater dissociation of electrolytes in the former or to mechanical separation of the fatty globules increasing the average width of the conducting paths. The want of effect of saponin on the conductivity of egg-yolk suggests again that in the ordinary cell it does not increase the conductivity

⁹⁴ *Zeit. für. physik. Chem.*, vi, p. 71, 1890.

⁹⁵ *Ibid.*, iii, p. 316, 1889.

⁹⁶ Hofmeister's *Beitr.*, vii, p. 393, 1906.

⁹⁷ *Journ. of Exper. Med.*, vol. vi, p. 257, 1902.

so much by diminishing the adsorption or chemical combination of electrolytes throughout the substance of the corpuscle as by favoring their passage through the envelope.

Membranes and Electromotive Phenomena.—As pointed out in my first paper, it is difficult to explain such phenomena as the vastly greater conductivity of nerve fibers in the longitudinal than in the transverse direction⁹⁸ except on the assumption that the membranes or envelopes, which cut the current lines in the latter case, interpose a great resistance to the passage of the ions. An adsorption or chemical combination of electrolytes in the interior of the fiber would act equally in diminishing the conductivity in whatever direction the current is allowed to flow. I do not think that anybody supposes that when nerve or muscle is stimulated electrically the current is conducted entirely by ions outside the fibers and not at all by the fiber contents. On the contrary, the most natural explanation of the inferior stimulating power of a transversely directed current is the smaller intensity of the current which traverses the contents of the fibers.

Guiffé,⁹⁹ under Hermann's direction, still obtained a marked difference in muscle even when he made the current density uniform by arranging two parallel-fibred muscles in series so that the current passed in the longitudinal direction through one and in the transverse direction through the other. He considered that he had in this way eliminated the difference of resistance as a factor in the difference between the longitudinal and transverse excitability. This, however, is not the case. For in transverse conduction much more of the current will traverse the interstitial tissue and much less the fiber contents than in longitudinal conduction, even though the average current density may be the same.

It is generally assumed that in the interior of the fibers free ions exist. They certainly exist in the vacuole contents of the cells which possess vacuoles. They exist, as already pointed out, in the contents of ova, where they are large enough to allow the question to be directly tested. They exist in the liquids expressed from all the tissues investi-

⁹⁸ Hermann, *Pflüger's Archiv*, v, p. 223, 1871; xxxix, p. 490, 1886.

⁹⁹ *Pflüger's Archiv*, xxi, p. 470, 1880.

gated and in the extracorpuseular liquid of colored corpuscles laked after washing away all the serum constituents with solutions of non-electrolytes like sugar. In short, the assumption that all the electrolytes in the colored corpuscles are so bound that they cannot conduct the current is opposed to all the direct evidence and is inferred simply from the fact that the corpuscles have a low conductivity, a phenomenon more simply explained by assuming that the envelopes which are known to exist in the case of certain kinds of colored corpuscles, at least, are not freely penetrated by ions. Nearly a dozen years¹⁰⁰ ago I pointed out the far-reaching relations of the low electrical conductivity of cells, whether due to badly conducting envelopes or not, in the explanation of many of the electrical phenomena of muscle and nerve, electrotonic currents and other so-called polarization phenomena, the high degree of polarizability of these tissues as compared with the polarizability of the surface of contact of ordinary solutions of electrolytes, etc.

In later papers¹⁰¹ it was pointed out "that the decided increase which I found in the potential difference between the belly and tendinous extremity of the frog's gastrocnemius, when sapotoxin solution (in salt solution) is applied to the muscle next the tendon, may be due either to a change in the permeability of the sarcolemma or to liberation of electrolytes in the fiber contents or to both. A similar though less marked increase is produced by sapotoxin in the current of rest of nerve." Also¹⁰² that the increase of conductivity which is known to occur in dying muscle may be due not only to increased formation of ions in the muscular substance, but to a change in the permeability of the muscular membranes. Numerous writers have since applied similar ideas to the detailed discussion of the electromotive properties of the tissues, including Macdonald, in a series of important papers¹⁰³ Bernstein;¹⁰⁴ Brünings;¹⁰⁵ Cremer;¹⁰⁶ Tschagonetz,¹⁰⁷ who refers to an

¹⁰⁰ Journ. Bost. Soc. Med. Sc.; Centralb. f. Physiol., loc. cit., 1897.

¹⁰¹ Amer. Journ. of Physiol., ix, p. 75, 1903.

¹⁰² Journ. of Exp. Med., vi, p. 259, 1902.

¹⁰³ Thompson-Yates Laboratory. Rep., p. 213, 1902, etc.

¹⁰⁴ Pflüger's Archiv, xcii, p. 521, 1902.

¹⁰⁵ Ibid., xcviii, p. 241; Ibid., C, p. 367, 1903.

¹⁰⁶ Zeit. für Biol., xlvii, p. 562, 1906.

¹⁰⁷ Ibid., l, p. 247, 1907.

earlier paper of his published in Russian in 1896. Quite recently R. S. Lillie¹⁰⁸ has made a valuable contribution on the relation of the "membranes" of contractile tissues to their electromotive phenomena.

The objection of Moore and Roaf¹⁰⁹ to the existence of a "membrane" which takes part in the regulation of the exchanges between the blood corpuscles and the plasma and especially in the regulation of the exchange of electrolytes is based, it seems to me, upon a special conception of the properties of the membrane. There is no *a priori* reason for denying to the envelope any of the powers of the protoplasm. When we have determined that a given substance passes easily, another with difficulty, into a corpuscle whose peripheral layer is histologically differentiated from its interior, we know no more about the mode of passage of the first substance, the cause of the hindrance of the passage to the second, than if the histological envelope had not been demonstrated. We have to inquire in the one case as in the other whether there is anything in the relative solubility of the two substances in the surface layer of the cell or in any of its constituents; whether there is any difference in the adsorptive power of that layer for the substances, or such a difference in its chemical affinity for them as will give the clue to the difference in the permeability of the cell to the two substances. We cannot say that it is "very unlikely that a membrane would allow hæmoglobin to pass out of the corpuscles while keeping back the electrolytes" unless the nature of the membrane in question is specified. Kahlenberg¹¹⁰ has indeed shown that the current view that crystalloids always pass through membranes more readily than colloids is untenable. Just the opposite may occur. It depends, as Tamann,¹¹¹ l'Hermite¹¹² and others showed long ago, upon the affinity of the substances for, or their solubility in the membrane.

Moore and Roaf¹¹³ made some interesting experiments on the passage of electrolytes from erythrocytes and from serum through dialyser membranes after treatment in various ways. They consider that these experiments afford evidence that the electrolytes in the corpuscles are

¹⁰⁸ Amer. Journ. of Physiol., xxii, p. 75, 1908.

¹⁰⁹ Bio-chem. Journ., iii, p. 55, 1907.

¹¹⁰ Journ. of Physical Chemistry, x, p. 141; Trans. Wisconsin Acad., Mar., 1906; Chem. Zentralblatt, i, p. 1391, 1906.

¹¹¹ Zeit. für. physikal. Chem., x, p. 255, 1892.

¹¹² Comp. rend., xxxix, p. 1177, 1854.

¹¹³ Loc. cit.

bound or adsorbed in such a way that they do not conduct, and that such compounds or adsorptates are kept back by the dialyser even after liberation from the corpuscles. Perhaps, however, they have not taken sufficient account of the retention of salts by the ghosts.

The increase of conductivity in these experiments when the corpuscles are laked by freezing and thawing is probably due to two things: (a) escape of a portion of the electrolytes and some water from the corpuscles; (b) the consequent diminution in the total volume of the corpuscles now represented by ghosts, with possibly some increase in the permeability of the ghosts to ions. The increase of the temperature to 40° C. for the conductivity measurement after the freezing and thawing might be expected to cause the liberation of a greater amount of electrolytes than in my observations, where the conductivity was measured at a much lower temperature.

The further increase in conductivity on dialysis against distilled water is due, mainly at least, to escape of a further quantity of electrolytes from the ghosts, under the influence of dilution, not to the breaking up of combinations still existing between the electrolytes and colloids after they have been liberated.

After the addition of ether to blood in quantity sufficient to cause rapid laking no ghosts could be seen, but only masses of granular débris.¹¹⁴ After water-laking probably every corpuscle is represented by a ghost. It is therefore easy to explain why more electrolytes were found in the dialysate after ether than after water, without assuming that adsorptates are broken up by the ether after liberation from the corpuscles. In the same way, the conductivity of the blood laked by saponin is further increased by heating the laked blood to the temperature at which heat itself causes laking. The final conductivity is about the same as when the same amount of saponin is added to heat-laked blood. The slight excess of conductivity in the former case is accounted for by the survival of fewer ghosts.

The final increase in conductivity on incineration is explained, as before, by the consideration that the ghosts even after water-laking still retain a considerable portion of salts. Doubtless, these salts are in part, at any rate, bound in the ghosts. The same explanation holds for the much smaller proportion of salts and the much smaller Δ of the dialysate from the corpuscles than of the dialysate from the serum.

That the stromata after certain methods of laking still remain relatively

¹¹⁴ Stewart, Journ. of Med. Res., viii, p. 283, 1902.

impermeable to certain salts, although the hæmoglobin has just passed out of them, follows from the fact that the ghosts shrink in hypertonic sodium chloride solutions as normal corpuscles do, and that the conductivity of a sediment of ghosts suspended in serum or sodium chloride solution is very small.

They occupy a far larger relative volume of the laked blood and, therefore, exercise a far greater effect on its conductivity than might be supposed from the comparatively scanty sediments which can be separated by the centrifuge. Nothing can be more inappropriate than the terms "rupture of the corpuscles," "breaking up of the corpuscles," "solution of the corpuscles," etc., often applied to the changes in hæmolysis. The laked blood is not a homogeneous solution. The main reason why its conductivity is not equal to that of a solution of the ash of the corpuscles and serum occupying the same volume is that much of the electrolytes is still in the ghosts, probably in the form of compounds of some kind. Another reason is that the ions of the serum and of the corpuscular contents already liberated cannot easily penetrate the ghosts. A certain portion of the electrolytes of the ash of course comes from lecithin and other compounds which are not electrolytes, by the oxidation of phosphorus, *e.g.*

The results of Roaf and Alderson¹¹⁵ on the dialysis against water of erythrocytes treated with chloroform, ether, acetic acid, etc., must be interpreted in view of the same considerations. In most, though not all, of their experiments, a larger amount of electrolytes dialysed out when the corpuscles had been treated in this way than when they were dialysed against water without treatment. This may be due, and very likely is due in part, to the setting free of electrolytes from combinations of some kind within the corpuscles and not to an alteration of the corpuscles which permits electrolytes already free to pass out of them. But the experiments do not prove this. For although it was seen that the corpuscles (dialysed against water) in the control experiments were completely laked, only their ghosts being left, this throws no light on the questions, how much of the electrolytes had left the ghosts and in what way the residue still within the ghosts was retained. A shadow laked by foreign serum has the same appearance under the microscope as a shadow laked by saponin. Yet the serum-laked ghost retains a much larger proportion of its electrolytes. The existence or the rôle of the envelope, if it does exist, cannot be determined in this way. The

¹¹⁵ Bio-chem. Journ., ii, p. 412, 1907.

fact, very well illustrated in these experiments, that electrolytes do leave the erythrocytes under the influence of anæsthetics may partly explain the increase in the osmotic concentration of the blood during ether and chloroform anæsthesia observed by Carlson and Luckhardt,¹¹⁶ although they consider that the main factor is the presence of the anæsthetic itself in the serum.

PART II. THE MECHANISM OF HÆMOLYSIS

Hæmochromolysis and stromatolysis. Adopting for the pigment in its natural condition within the corpuscle Bohr's term "hæmochrome," I conceive of hæmolysis as follows: We have to distinguish in this process the mere liberation of the blood pigment, or hæmochromolysis, from the more or less complete destruction or solution of the stromata, stromatolysis. Hæmochromolysis *plus* stromatolysis constitutes erythrocytolysis. Unfortunately the term hæmolysis has been applied indifferently to any process which causes liberation of the blood pigment from the corpuscles, being equivalent sometimes to hæmochromolysis, sometimes to erythrocytolysis.

Hoppe-Seyler¹¹⁷ considered the native blood-pigment in the corpuscles to be a compound of hæmoglobin with lecithin. Now in the body lecithin shows a marked tendency to unite with other substances, including proteins.¹¹⁸ So that we may perhaps conceive of the hæmochrome complex as a hæmoglobin-lecithin-protein combination, a combination which is very easily broken up so as to yield hæmoglobin. Bang¹¹⁹ has shown that lipid substances, especially bodies like lecithin, may under certain conditions be fixed by hæmoglobin, possibly in the form of adsorptates.

Sjövall,¹²⁰ in his study of the staining reactions of the nerve cells and fibers with osmic acid, has brought forward evidence that water breaks up a loose compound between the lipoids and the rest of the cell contents. And Albrecht;¹²¹ quoted by Bang,¹²² states that a similar decomposition

¹¹⁶ Amer. Journ. of Phys., xxi, p. 162,

¹¹⁷ See Bohr's article in Nagel's Handbuch der Physiologie, i, p. 90.

¹¹⁸ See Glikin in Oppenheimer's Handbuch der Bio-chemie., i, p. 128, 1908; Bang, Ergebnisse der Physiologie, Jahrg. vi, p. 136, 1907.

¹¹⁹ Loc. cit., p. 152.

¹²⁰ Anat. Hefte, 1905.

¹²¹ Verh. der Anat. Ges., 1902.

¹²² Ergebnisse der Physiol., vi, 1907.

resulting in the setting free of lecithin in the protoplasm of various cells, takes place spontaneously after death.

The essential thing in liberation of the blood pigment is the change of the hæmochrome, which exists in colloid or solid solution or perhaps as an adsorbate in the stroma or as a weak chemical combination with the stroma, into an aqueous solution of hæmoglobin, or, at any rate, into an aggregate condition which by comparison with its normal condition in the corpuscles may be denominated water-rich. Given such an aqueous solution of hæmoglobin in the interior of the corpuscle, its liberation follows inevitably. Water-soluble hæmoglobin never exists in the corpuscle till the moment of laking arrives. It is foreign to the corpuscles and is promptly extruded. The mechanism of this expulsion of the hæmoglobin is certainly an interesting subject of study and to that we shall return. But let it be repeated, on our view the essential thing is the alteration of the natural blood pigment in the interior of the corpuscle. All the fundamental phenomena of hæmochromolysis may indeed occur, as in *Necturus* corpuscles, without liberation of the hæmoglobin from the corpuscle. The hæmoglobin of these corpuscles crystallizes so readily that by proper graduation of the dose of hæmolytic agents the hæmoglobin liberated in their interior may be crystallized before it can escape. The entrance of such an amount of water from the suspending liquid as will dissolve the crystals, or perhaps the liberation of a sufficient amount of the intracorpuseular water, leads to the instant escape of the hæmoglobin.

Löffler's methylene blue (alcohol action) causes intraglobular crystallization without swelling of the corpuscles or nuclei. Here the crystallization is not determined by increase of water in the corpuscles. What determines it? Perhaps precipitation of proteins, which allows the hæmoglobin to separate from the stroma, perhaps the solution of lipoids. Occasionally *Necturus* corpuscles become globular under the influence of Löffler's methylene blue, and these do not show intraglobular crystallization. Probably the explanation is that when the alcohol alters the envelope in such a way that much water penetrates it the hæmoglobin passes into solution and is then liberated, whereas the entrance of alcohol without upon the whole any water having gone into the corpuscles causes such a change that hæmoglobin crystals are formed. Intraglobular crystallization in partially dried

Necturus blood may be due to the entrance of water through the altered envelopes of the corpuscles. An alteration of the envelopes such as permits the escape of hæmoglobin is therefore not the primary phenomenon in hæmolysis, but rather an alteration in the nature or in the relations of the blood pigment within the corpuscle.

Escape of the hæmoglobin solution through the envelope may be due to (1) the altered molecular condition of the stretched envelope when the corpuscle is swollen by the entrance of water; (2) a chemical or physico-chemical action of the hæmoglobin or the water on the interior surface of the envelope. J. Traube¹²³ states that hæmoglobin has a hæmolytic action, and it may therefore be supposed to act on or be taken up by some constituent of the envelope. In virtue of this action the hæmoglobin passes through the envelope, coming into contact at the outer surface of the envelope with water or a saline solution, in which it readily dissolves. If it is lytic for the corpuscle it is easy to see why the existence of an aqueous solution of it in the interior of the corpuscle is incompatible with the integrity of the latter. The point to be noted is that hæmoglobin is a foreign substance to the corpuscle, and there is no evidence that it can be regenerated to hæmochrome. Its liberation may therefore be a phenomenon belonging to the same group as the excretion of hæmoglobin from the blood by the kidney when it exists in sufficient amount in solution in the plasma. Only, in the kidney the hæmoglobin affects the cells in such a way that it passes from its solution into the cells, whereas in the case of the laked corpuscle it passes from solution in the cell contents through the substance of the cell into the blood plasma. It is certain, however, that the liberation of the hæmoglobin from the corpuscles in the act of laking is not a matter of mere diffusion. For the ghosts separated from laked blood contain much less hæmoglobin than an equal volume of the suspending liquid. And ghosts once completely freed from hæmoglobin do not take it up again from the hæmoglobin-containing liquid. It might be supposed that the affinity or the adsorptive power of the stroma for hæmoglobin had been lost by the passage out of the corpuscles of the constituents by which the blood pigment is bound or adsorbed within them. Or, again, the hæmolytic power of

¹²³ Traube and Clara Goldenthal, *Bio-chem. Zeit.*, x, p. 390, 1908.

hæmoglobin might be supposed to prevent the ghosts from re-accumulating that pigment. This, however, is unlikely because the hæmolytic power of hæmoglobin, acting outside the corpuscles, is comparatively slight, so slight that hæmolysis may remain incomplete for many hours in the presence of a large amount of hæmoglobin in the solution. Further, the hæmolytic action of the blood pigment is antagonized by serum (an antagonism which, of course, would not affect hæmoglobin acting in the interior of the corpuscles), yet ghosts take up no more hæmoglobin from a serum solution of hæmoglobin than from an aqueous solution. The most probable explanation of the failure of ghosts to take up hæmoglobin is that the envelope has no affinity for this substance. In the intact corpuscle it is free from hæmoglobin for this reason, and normal corpuscles do not take up hæmoglobin from a solution of it in serum, a fact which I have made the basis of a method of estimating the relative volume of corpuscles and plasma in the blood.¹²⁴ There is nothing surprising in the fact that hæmoglobin in the process of laking should pass out of the corpuscle through the envelope, while after laking it will not pass in the reverse direction. For, firstly, the hæmoglobin in the one case is in solution in blood serum, or other suspending liquid; in the other case, in solution in the contents of the corpuscle, or in some other relation to them than that of a solution.

Irreciprocal permeability for a given substance may just as well be produced by a difference in the condition of the substance on the two sides of a homogeneous membrane as by a difference in the nature of the two surfaces of a membrane exposed to identical solutions.

In the second place, if the envelope of the cell is histologically differentiated on the side of the cell contents, it might be expected that in accordance with the law of W. Gibbs recently used by Traube in the discussion of hæmolysis, the external and internal surfaces of the membrane should exhibit physico-chemical differences leading to irreciprocal permeability even of a substance in similar solution in contact with these two surfaces. For the passage of substances from the plasma into the outer surface of the envelope and of substances from the cell contents into its inner surface would render the two surfaces eventually heterogeneous.

¹²⁴ Journ. of Physiology, xxiv, p. 356, 1899.

Thirdly, there is reason to believe that the condition of the envelope of the corpuscle does not remain the same as regards its physico-chemical properties during the whole process of laking. For example, at the time when hæmoglobin is about to pass out, in some forms of laking, it seems that the permeability of the envelope to certain ions is increased, to diminish again after the liberation of the hæmoglobin.

This is shown by the fact that after various forms of laking the swollen ghosts are caused to shrink by the same hypertonic solutions as cause shrinking of the intact corpuscles. Even when the corpuscles in the act of laking swell up in hypertonic solutions in which they were previously crenated, the very same hypertonic solutions may cause the ghosts to shrink. It is not quite the same envelope, then, through which the hæmoglobin makes its exit in laking and which it refuses to enter after the laking is finished.

The question whether it is possible for stromata from which hæmoglobin has been liberated by the gentlest means—that is, with the least possible alteration of the stromata themselves—to regenerate hæmochrome, does not enter into the discussion. If they could do so, we might expect the expulsion of the hæmoglobin to be the first step, or, rather, an essential preliminary to regeneration of the blood pigment within them. But whether they possess this power or not, they certainly did as erythroblasts possess the power of forming hæmochrome, and the getting rid of the hæmoglobin might be looked on as an attempt on the part of the protoplasm of the erythrocytes to put itself once more in the position of the colorless erythroblasts before the formation of the hæmochrome began. The absence of nuclei in the mammalian corpuscles, even if we assume the absence of diffused nuclear substance, is not a relevant objection.

As regards the structure of the erythrocyte, in its relations to hæmolysis, a brief recapitulation of our conception¹²⁵ may be permitted. The corpuscles consist of a protoplasmic groundwork bounded externally by an envelope. In large corpuscles, like the *Necturus* corpuscle, this envelope is better differentiated than in mammalian corpuscles and, therefore, more easily demonstrated histologically. The envelope and stroma consist essentially of lipoids (lecithin,

¹²⁵ Cf. Peskind, Amer. Journ. of Med. Sciences, p. 1011, 1904.

cholesterin), proteins (nucleoproteins), with of course, water and salts. The interior stroma, but not the envelope, is impregnated with blood pigment, either loosely combined or adsorbed by it. When gentle hæmolysis occurs, a change is produced in the envelope by the action of the hæmolytic agent on one or more of its constituents. This change permits the entrance of a certain amount of the hæmolytic agent as well as in general a certain amount of water. By the action of the hæmolytic agent (or the water) on the stroma, the relation of the blood pigment to it is altered so that the pigment goes into aqueous solution or becomes water-soluble. This is aided in most cases by the entrance of water into the corpuscle from the suspending liquid. The envelope allows hæmoglobin in watery solution to pass out through it. As shown by O. Pascucci¹²⁶ an aqueous solution of hæmoglobin cannot be retained by an envelope consisting of cholesterin or of lecithin and cholesterin after the action of saponin, solanin, cobra poison, etc.

The objection of Pascucci¹²⁷ that the lipoid content of the corpuscles is too high to allow us to suppose that the lipoids belong to a stroma, a protoplasmic groundwork filling up the corpuscle, and that the analogy is rather with a sheath, like the medullary sheath of nerves, proves nothing. In the development of the hæmoglobin, it is to be supposed that the globin represents a great part of the original protein content of the protoplasm, the pigment portion of the molecule being everywhere distributed uniformly in combination with the globin. It results from the fact that so large a proportion of the original protein of the protoplasm is taken up in forming the globin, that the proportion of lipoids in the stromata appears high. Distributed over the whole protein of the corpuscle it is not so. Pascucci's objection to the existence of an interior stroma, that when intraglobular crystallization is induced we see only crystals and nothing between them, is not cogent. The material between them is colorless, and after the hæmoglobin has separated must certainly be rich in water. It is easy to see that when partially fixed corpuscles are laked, the interior of the corpuscle is not empty even when all the blood pigment has passed out. If the globin represents a part of the original protoplas-

¹²⁶ Hofmeister's Beitr., vi, p. 552, 1905.

¹²⁷ Ibid., p. 543, 1905.

mic protein, it is easy to see that through it the hæmoglobin molecule may be anchored to the stroma, the globin having affinities both with stroma and with hæmochromogen. When water enters the corpuscle in more than a certain amount the hæmoglobin—in virtue of its solubility in water—parts company with the stroma, the chief constituents of which are insoluble in water, and thus the hæmochrome complex is ruptured. In like manner, if a substance like saponin which is capable of dissolving lipoids but not proteins or hæmoglobin enters the corpuscle in sufficient amount, the lipoids of the stroma go into solution, thus parting company with the hæmoglobin. It may be that till the moment when separation between the hæmoglobin and the stroma arrives, the hæmoglobin molecule does not exist as such, but only as a complex containing the pigment hæmochromogen, the globin and the stroma constituents with which the globin is united.

When the complex decomposes under the influence of ordinary hæmolytic agents which do not act destructively upon the blood pigment, hæmoglobin is always formed because the affinity of the globin for the hæmochromogen is stronger than for the stromata.

Comparatively little is known about the chemistry of globin, and especially about the nature of the union between it and the pigment part of the hæmoglobin molecule on the one hand and the stroma on the other. It is a histon, and as such soluble in water, from which it is precipitated by especially small quantities of ammonia. It is then dissolved with special ease in excess of ammonia. Ammonium salts hinder its solution in not too great quantities of ammonia.¹²⁸ With acids histons form salts, which in the case of the inorganic acids are soluble in water. It is possible that these reactions are concerned in laking by alkalis and acids as well as in water laking.

Hæmochromolysis, then, may be produced in two ways: by dissociating the complex, by exposing it to water—that is, by an increase in the water content of the corpuscle—or by dissolving the stroma without necessarily increasing the water content. In the case of many laking agents the two factors act together. A typical instance of such agents is saponin, which increases the permeability of the corpuscles to ions by acting on the lipoids of the envelope, and thus per-

¹²⁸ Samuely, Oppenheimer's Handbuch der Bio-chem., i, p. 306, 1908.

mits water to enter, while at the same time, if the dose is sufficient, it acts directly on the stroma-hæmoglobin combination. When just enough saponin is used to liberate all the hæmoglobin, the action may be considered as approximating to a pure water action. That is to say, the minimal dose of saponin need not cause any other change than the increased permeability of the corpuscle. Laking will follow from this alone. This is the condition in which a relatively small quantity of electrolytes leaves the corpuscles with the hæmoglobin. If more saponin be added to the ghosts a further action takes place which, under certain circumstances, breaks up the ghosts completely and under all circumstances causes a relatively large escape of electrolytes from them. With a supraminimal dose of saponin added to the corpuscles at once, the two actions go on together.

Water itself acts also in both ways, since the addition of it to stromata laked by gentle means results in the liberation of a relatively large amount of electrolytes. Whether the water passes into the corpuscles on account of difference of osmotic pressure, in the orthodox sense, or by reason of an affinity for water on the part of constituents of the envelope or stroma, it certainly acts on the envelope, as shown by the agglutination which precedes hæmolysis. It may do this either by producing imbibition of the lecithin or by acting on the nucleo-protein. A similar action on the whole stroma of the already gently laked corpuscle may take place, one sign of the decomposition being the setting free of electrolytes.

That there is something in the nature of a stroma in which the blood pigment is distributed is well illustrated by the behavior of sublimate- or formaldehyde-fixed corpuscles when caused to swell and shrink after a certain degree of fixation has been reached. The same observations show that the blood pigment need not be assumed to be in solution in the corpuscles in order to explain its uniform diffusion throughout the stroma, whether the corpuscle is caused to swell or shrink. At a time when the fixation has gone so far that none of the pigment escapes from the swollen corpuscle, the tint of the corpuscle remains perfectly uniform. There is no appearance of granulation of the pigment. Yet, on rubbing up the corpuscles thoroughly with sand no trace of pigment passes into the suspending liquid, although microscopic examination shows that the corpuscles have been triturated

into fragments. The pigment does not go into solution although brought into contact with an aqueous liquid because it has been rendered insoluble in water. Yet, when the corpuscle swells, the pigment remains equally distributed over it and it appears exceedingly pale. When the corpuscle shrinks the pigment assumes its original distribution and tint. The process reminds one of the paling and pigmentation of frog's skin when the pigment cells contract and relax. It is difficult to see how a mass of precipitated hæmoglobin or met-hæmoglobin such as we know them outside the corpuscles would continue to be quite uniformly distributed when the corpuscle is swollen and would return to its original and still uniform concentration when the corpuscle was made to shrink to its original volume. One would have expected rather, if the mass of blood pigment was simply enclosed in an envelope which in virtue of osmotic changes became first distended and then retracted, that a separation of the pigment from the envelope would take place during the distention. Nothing of the kind, however, occurs.

A certain amount of electrolytes accompanies the hæmoglobin in its passage out of the corpuscle, either electrolytes which are normally in aqueous solution in the cellular liquid or electrolytes separated from the stroma at the same moment as the hæmoglobin and going into aqueous solution along with the pigment, or, possibly, electrolytes split off later from the stroma by the dissociating influence of the water which has entered. If the action of the hæmolytic substance is just sufficient to cause liberation of all the hæmoglobin, a great part of the electrolytes of the corpuscles still remain in the stroma. The electrolytes which still remain may represent: (1) that portion which is more firmly bound to the stroma than the portion which escapes, constituting as it does the electrolytes necessary for the physiological condition of the protoplasmic groundwork; (2) a portion of the electrolytes in aqueous solution which is unable to escape either because the envelope is impermeable to them throughout the whole process of laking, or because, although permeable while the corpuscle is still swollen, it becomes impermeable again after the discharge of the hæmoglobin. Such a change in the permeability of the corpuscle is illustrated by the fact that in the process of formaldehyde-fixing the corpuscle at first becomes more permeable than normal to sodium chloride, but later on regains its original impermeability.

When the action of the laking agent is more intense, electrolytes are split off from the stroma and escape through the still more altered envelope and electrolytes already in solution in the ghosts, but unable to penetrate the envelope, now become capable of doing so. For reasons given in Part I the conclusion was drawn¹²⁹ that "the increase of permeability of corpuscles produced by saponin is probably caused by a corrosive, dissolving or emulsifying action of the saponin on some non-protein constituents of the envelope or stroma." Evidence was also obtained that "in the first stage of the action of saponin on unfixed blood there is an increase in the permeability of the corpuscles for ions even before any hæmoglobin has been liberated." It was suggested that "the liberation of the hæmoglobin may be secondary to this owing to the entrance of water consequent on the disturbance of osmotic equilibrium."¹³⁰ I showed, however,¹³¹ that although the increased permeability of the corpuscles, which entails the entrance of water, is a stage, and doubtless an important one, in many kinds of hæmolysis, it is not the whole process.

For, firstly, laking of crenated corpuscles may take place without swelling under the influence of sodium taurocholate. Secondly, laking may take place on the addition of any of the ordinary chemical hæmolytic agents, in substance, to a sediment of corpuscles with so small a quantity of serum between them that even if the whole of the water of the serum enters the corpuscles it would not be sufficient to produce ordinary water laking.

The red corpuscles are exceedingly poor in water compared with other cells, the solids constituting more than 40 per cent of the total mass, whereas in thymus leucocytes according to Lilienfeld, the solids make up only 11.5 per cent of the whole. So far as I know, the water content of erythroblasts before development of the blood pigment has not been determined. Yet we can assume that in developing the blood pigment in their interior the erythroblasts in all probability become poorer in water. It is an interesting speculation whether this has any relation to their function. There may be two reasons—one functional, the other physico-chemical: (1) to increase the oxygen-

¹²⁹ Journ. of Physiology, xxvi, loc cit., Journ. of Exp. Med., vi, p. 257, 1902.

¹³⁰ Amer. Journ. of Physiology, ix, loc cit.

¹³¹ Journ. of Med. Research, loc cit.

binding capacity of unit volume of the blood and therefore to facilitate rapid aëration in the lungs and rapid giving up of oxygen in the tissues without too great a blood velocity; (2) to permit the existence of the blood pigment as a gel. It seems probable that a body so soluble in water as hæmoglobin would pass into aqueous solution, or a body so capable of dissociation as hæmochrome in the presence of water would be dissociated so as to yield hæmoglobin, if the water-content of the erythrocytes were as high as that of the leucocytes. As a matter of fact, when an amount of water considerably less than that required to increase the water content of the erythrocytes to an equality with that of leucocytes is introduced into them, the hæmoglobin passes into aqueous solution and is discharged. In the case of *Necturus* corpuscles a still smaller quantity of water causes the transformation of the hæmochrome *in situ* into crystalline hæmoglobin, which is only liberated from the corpuscles when the further amount of water necessary to dissolve the crystals is introduced.

In the replacement of the hæmochrome gel by the hæmoglobin sol or solution, I see a decisive event in hæmolysis. Complete hæmochromolysis may, however, not be accompanied by marked stromatolysis; in part this depends on the quantity of the hæmolytic agent employed, in relation of course to the sensitiveness of the corpuscles, in part on the nature of the hæmolytic agent. Some laking agents, even in quantities more than sufficient to liberate all the hæmoglobin, do not destroy the stromata nor even set free any great amount of stroma electrolytes. Such are the biological laking agents. Others, even in amounts only sufficient to liberate all the hæmoglobin, seem to produce considerable changes in the stroma, characterized for one thing by the liberation of stroma electrolytes. Such an agent is water. Others still, like saponin, heating, freezing and thawing, produce complete liberation of the hæmoglobin, while the stroma is not much affected, if a strictly minimal action has been sought for; but marked effects are produced on the stroma when the action is allowed to become more intense.

It is perhaps the poverty of the erythrocytes in water which renders them so sensitive to the action of hæmolytic agents, since so many of these are known to cause the passage of water into the corpuscles as a preliminary to laking. Even where an artificial erythrocyte,

like a formaldehyde-fixed corpuscle, is laked by heating in ammoniacal water, or a formaldehyde corpuscle treated with ammonia and then washed till ammonia ceases to be given off is simply heated in water, marked swelling of the corpuscles (entrance of water) is necessary before laking takes place. The slight drying of the corpuscle which is necessary to determine laking in the presence of extracorpuseular water in the form of solutions which do not lake normal corpuscles is another proof that the erythrocyte exists in a state of unstable equilibrium as regards water, even if the limits within which the water-content may vary are comparatively wide.

The normal range of "osmotic resistance" of the corpuscles may be not much greater than the range within which the osmotic pressure of the plasma in particular capillaries may vary even in health. In the kidney, for example, where a secretion whose molecular concentration is in general much greater than that of the plasma is formed, the molecular concentration of the plasma on the whole must sink. The diminution may be very considerable in particular renal capillaries or capillary tracts. During absorption of water from the intestine the same thing may occur. In disease it may be expected that such changes should be exaggerated, and it is possible that various forms of hæmoglobinæmia may be due to a hæmolysis thus caused or favored. On the other hand, in the salivary glands during active secretion a liquid of much lower molecular concentration than the plasma is separated and therefore here the molecular concentration of the plasma must increase. The changes in the plasma of blood drawn from a large vessel will, of course, be no index to the possible changes in special capillary tracts. It is plain, then, that the colored corpuscles must be fitted to withstand considerable variations in the osmotic pressure of the plasma. This is in addition to the changes produced by alterations in their permeability occasioned, for instance, as Hamburger has shown, by variations in the carbon dioxide content of the blood in different regions. Undue stress has been laid on the relative constancy of the average concentration of the blood plasma, a constancy which tends to obscure the continual and rapid vicissitudes of osmotic pressure experienced by the corpuscles as they pass along the vascular system. If the velocity of the blood stream is pathologically diminished in any portion of the kidney or intestines while the excretion of

solids or the absorption of water is not, or is not so much interfered with, the permissible limit of hypotony may be passed and intravascular hæmolysis may result. Since in water-laking agglutination precedes the liberation of the blood pigment, it is conceivable that local thromboses may be caused in this way. It is possible that the older erythrocytes may normally succumb in such regions of low osmotic pressure.

In his studies on *Bothriocephalus anemia* Tallquist¹³² obtained from the proglottides of the worm a substance of strong hæmolytic properties and the chemical characters of a lipid. This substance, according to his statements, caused in animals slight but distinct anemia with the characters of pernicious anemia. This seemed to strengthen the position of those who hold that progressive anemia is due to an enterogenous autointoxication. However, E. Bloch¹³³ failed to obtain from the feces of a person suffering from progressive anemia any substance corresponding in its action to Tallquist's lipid. Indeed, the substances extracted by ether or alcohol from the feces of a healthy person possessed the stronger hæmolytic action. It is quite possible that substances which produce these forms of anemia, if they are derived from the bowel, may exert their hæmolytic action, or a part of it, indirectly by injuring, in the first instance, the intestinal epithelial lining, rendering it more permeable than normal to water in the direction from the lumen to the blood and lymph, or more permeable than normal to dissolved substances in the direction from the blood or lymph to the lumen of the gut. In either case such areas of hypotony as have been suggested would be produced in certain vascular tracts in the intestinal wall. In paroxysmal hæmoglobinuria vasomotor changes associated with exposure to cold may conceivably give rise to such hypotonic regions, in which, of course, the hæmolytic action of the serum (plasma) studied by Donath and Landsteiner,¹³⁴ by Hoover and Stone¹³⁵ and by others, would be reinforced by the hypotony.

The action of salts and other substances, like cane sugar, which do

¹³² Zeit. für. Klin. Med., lxi, cited by Bloch.

¹³³ Bio-chem. Zeit., ix, p. 498, 1908.

¹³⁴ Münch. Med. Woch. lxi, p. 1590, 1904.

¹³⁵ Archives of Internal Medicine, Nov. 1908.

not easily penetrate the corpuscles, in inhibiting certain kinds of hæmolysis, for example, hæmolysis by foreign serum, is probably in part an osmotic action which hinders the entrance of water into the corpuscles, the water being unable to enter from hypertonic solutions except in company with the dissolved substances. There is nothing in this inconsistent with Höber's view that certain neutral salts may exert an action on the corpuscles which alters their permeability by altering the physical condition of the colloids in the superficial layer. Nor is there anything inconsistent with my observation that blood charged with ammonium chloride, a substance which readily penetrates the blood corpuscles, is much less easily laked by foreign serum or saponin than normal blood. The circumstance that the foreign serum in this case brings electrolytes (doubtless ammonium chloride) out of the corpuscles in preference to hæmoglobin, whereas normally it brings out hæmoglobin in preference to electrolytes, is instructive. Here we have a corpuscle whose stroma is enriched with a salt which there is reason to believe is not all simply in solution, but partly bound or adsorbed, perhaps in much the same way as the blood pigment itself, but more loosely. The lytic serum increases the permeability of the corpuscles and water enters them. But instead of dissociating the hæmoglobin from the stroma, we may suppose that the water first dissociates the less firmly held salt, which goes into aqueous solution and in this condition easily passes out of the corpuscles. Later on, the water accomplishes the solution of the hæmoglobin, but more slowly and incompletely than in the absence of the salt. The condition of a portion of the ammonium chloride in the corpuscle, seems to be analogous to that of the relatively small amount of electrolytes which escape from the corpuscle with or before the hæmoglobin in the laking of normal corpuscles by the more gentle hæmolytic agents. Some of the ammonium chloride may be more firmly united to the stroma, and saponin produces the same marked increase in conductivity as it does in normal blood.

In Table X of the paper cited,¹³⁶ the conductivity of rabbit's ammonium chloride blood after the action of dog's serum was 106.5. If simple mixture had taken place without redistribution of electrolytes, it

¹³⁶ Journ. of Physiology, xxiv, p. 228, 1899.

would have been only 92.3. The difference is not due to mere dilution of the blood with serum, since in Table IX the conductivity of normal rabbit's blood after addition of dog's serum is less than it would have been if simple mixture had taken place, even before escape of the hæmoglobin, probably because electrolytes have entered the corpuscles. When the osmotic equilibrium is upset by the hæmolytic action of the foreign serum the corpuscles, abnormally rich in electrolytes, and in electrolytes which easily penetrate the envelope, lose a portion of their excess, while corpuscles containing the normal electrolytes in normal amount tend at first rather to gain electrolytes from the serum. This agrees very well with the idea that normally the osmotic concentration in the interior of the corpuscles is somewhat less than that of the serum, a condition which may account for the transitory shrinking of the corpuscles in serum hæmolysis observed by Baumgarten.

Hæmolytic experiments on artificial or denatured erythrocytes. In order to study the complicated phenomenon of hæmolysis by eliminating certain of the factors, one may have recourse to corpuscles more or less altered, for instance, to corpuscles fixed in various ways, especially when the nature of the fixative process and the point of attack of the fixing reagent are more or less understood. So far as I am aware, this method was first employed by me.¹³⁷ I used formaldehyde to fix the corpuscles, also heat, osmic acid, and other agents. Matthes¹³⁸ employed corpuscles fixed by Hayem's solution (sublimate) in certain studies on hæmolysis. Sachs¹³⁹ showed that substances like potassium iodide and sodium hyposulphite, which can combine with mercury, will laked sublimate-fixed corpuscles, and that serum, whether heated or not, whether foreign or belonging to the same animal, possesses this property also in virtue of the power of its proteins to unite with the mercury salt and to withdraw it from its combinations in the corpuscles. Von Dungern and Coca¹⁴⁰ have recently announced that osmic-hardened corpuscles can be laked by foreign sera. In formaldehyde fixing, as already mentioned, the erythrocyte is altered so little in certain important respects that we are entitled to look upon corpuscles fixed in this way as cells whose envelopes are still exceed-

¹³⁷ Journ. of Physiology. xxvi, p. 494, 1901.

¹³⁸ Münch. Med. Woch., Jan. 7 and Apr. 29, 1902.

¹³⁹ Ibid., Feb. 4, 1902.

¹⁴⁰ Berlin. Klin. Woch., xlv, p. 1471, 1907.

ingly good imitations of the envelopes of normal corpuscles—much better imitations, it is hardly necessary to say, than those cholesterol-lecithin membranes with which Pascucci worked¹⁴¹ and on which he obtained results of some interest. The hæmoglobin of the formaldehyde-fixed corpuscles is ultimately changed into methæmoglobin. The hæmoglobin molecule is not disrupted with formation of hæmatin if only the amount of formaldehyde necessary for fixation be employed in sufficiently dilute solution, but it is probably precipitated along with the proteins of the stroma.

The formaldehyde-fixed corpuscles are not laked on suspension in distilled water, even on heating, nor by the action of any of the ordinary laking agents, but can be laked by heating in ammoniacal water. Sublimate-fixed corpuscles at a certain stage of fixation do not lake in distilled water at room temperature, but do so in ammoniacal water, and are laked in distilled water on heating. Both in sublimate and in formaldehyde fixation a stage can be found at which apparently only the peripheral portion of the corpuscle has been hardened, the blood pigment in the interior remaining little, if at all, altered. We have, then, in the fixed corpuscle a simplified scheme of an erythrocyte on which we can study certain questions more readily than on the unaltered corpuscles. For instance, the permeability of the corpuscles to various substances can be investigated with the view of determining, uncomplicated by the escape of hæmoglobin or stroma constituents, whether the normal properties of the corpuscle in this regard are retained. I have shown that saponin is fixed by formaldehyde corpuscles from solution at 0° just as by the normal corpuscles. Also that ammonium chloride penetrates the formaldehyde corpuscles and sodium chloride does not or to a much smaller degree, as is also the case in normal corpuscles. Ammonium chloride seems to be retained in a manner similar to that in which it is retained in the normal corpuscle. After the action of saponin, which renders the formaldehyde corpuscle freely permeable to sodium chloride as well as to ammonium chloride, no binding of the latter seems to occur. This is also true of the ghosts of normal corpuscles laked by saponin.

Since formaldehyde corpuscles acted on by saponin are equally per-

¹⁴¹ Loc. cit.

meable to sodium chloride and ammonium chloride, and the saponin action is a surface action, or at any rate is not associated with the setting free of electrolytes in the interior of the corpuscles, the selective permeability of formaldehyde corpuscles for ammonium chloride is largely an affair of the envelope. It is at any rate not due to an action of the formaldehyde or the formaldehyde-protein compounds, since there is no reason to believe that these are acted on by saponin. No difference between sodium chloride and ammonium chloride in the case of heat-laked blood, either mammalian or fowl's, was observed. In the case of saponin-laked and water-laked corpuscles, however, the characteristic difference between sodium chloride and ammonium chloride still persisted, the former shrinking the ghosts, the latter not. The peculiarity of the corpuscles on which the selective permeability depends is therefore a property of the stroma (with its envelope), a property which is destroyed by exposure to the temperature at which heat-laking occurs. But the experiments do not exclude the blood pigment *in situ* from a share in the action.

In the fixed corpuscle the relation of blood pigment to stroma must of course be very different from the normal. But although the combination is artificial, it is none the less interesting to ask the question whether and how liberation of the blood pigment is brought about. The conditions of liberation of the blood pigment when it exists in a more or less known state in the corpuscle may be expected to throw light on the state of the pigment in the normal corpuscle, from which it can be liberated by establishing certain known conditions. The formaldehyde itself, in such concentrations as were used for fixing, causes at first a small amount of laking. During this period the corpuscles can be shown to be more permeable than normal, not only to sodium chloride, but also to ammonium chloride. Later on, as fixing proceeds, the corpuscles again become as impermeable to sodium chloride as normal corpuscles, while still remaining relatively permeable to ammonium chloride. It is probable that the same action of formaldehyde which causes the slight preliminary laking causes the increase of permeability to sodium chloride, an increase of permeability which in the sodium chloride suspensions conspires to increase the hæmolysis by permitting the entrance of water into the corpuscles. When the corpuscles are fully fixed, as has been said,

they are incapable of being laked by ordinary procedures. Yet if the corpuscles be suspended in water and treated with dilute ammonia slow laking occurs at ordinary temperature, while on heating to 60° C. or, indeed, to a lower temperature, rapid and complete liberation of the blood pigment ensues. What is the mechanism of hæmolysis in this case? It might be thought, that the same change occurs as in the normal corpuscles which are hæmolyzed by alkalies. But this is not an ordinary alkali action, for it is obtained far less easily with sodium hydroxide, although sodium hydroxide is a stronger base and lakes ordinary corpuscles in a smaller concentration than ammonia. Nor is it due directly to the action of ammonia on the blood pigment. For both ammonia and sodium hydroxide change the methæmoglobin and alter the color and spectrum of the blood pigment in precisely the same way and with equal ease. Further, the change in the color of the blood pigment is not an essential condition of the laking. For, if after addition of ammonia the corpuscles be washed with water till the washings give no alkaline reaction to litmus, the blood pigment reverts to its original brown color and shows again the spectrum of methæmoglobin. The corpuscles have exactly the appearance of ordinary formaldehyde-fixed corpuscles. They differ essentially however in this,—that they can now be laked completely by heating in water without further addition of ammonia. Either, then, ammonia still remains fixed in some constituents of the corpuscles, although not affecting the blood pigment, or it has produced a change in some of the corpuscular constituents which is permanent after the excess of ammonia has been washed out. When formaldehyde corpuscles are tested at intervals during the process of fixation, it is found that at first they are easily laked in water alone at ordinary temperature. Later on water does not cause laking at the ordinary temperature, but does so when the temperature is raised. It is not necessary that the temperature should be raised to that of heat-laking of unfixed blood corpuscles. Complete liberation of blood pigment has been obtained at 40° C. in a few minutes. Still later the corpuscles become incapable of being laked in water at any temperature. At this stage the addition of ammonia will cause laking in water at the ordinary temperature. At a still later stage in the fixation process not only must ammonia be added, but the temperature must be raised in order to obtain libera-

tion of the blood pigment. Finally even ammonia and heat will only cause swelling and paling of the corpuscles without liberation of the pigment. At this stage ammonia still easily penetrates the corpuscles as is shown by the instant change in color from brown to red when it is added. For a considerable time, as fixation goes on, although the color of the suspension is distinctly brown, the spectrum shows strong oxyhæmoglobin bands along with a methæmoglobin band in the red of moderate strength. The cautious addition of ammonia causes the band in the red to disappear without any effect on the oxyhæmoglobin bands. If more ammonia be added, a new band makes its appearance just to the left of the D line. The most likely interpretation of these appearances is that the formaldehyde, penetrating into the corpuscles, first changes the more superficial portion of the blood pigment into methæmoglobin, while the interior portion for a time remains unchanged. If this be true, then at this stage we have a corpuscle in whose interior the normal blood pigment still exists, but is not liberated by the action of water because water cannot now produce the changes in the external layer associated with the water-laking of the normal corpuscle. In other words, the partially fixed formaldehyde corpuscle is an instance of a corpuscle protected against ordinary laking agents by an alteration in the superficial layer. If, now, at a sufficiently early stage of fixation, we act on the corpuscle by ammonia, it is restored as regards water-laking to the position of a normal corpuscle. The ammonia in this case affects the partially fixed peripheral layer of the corpuscle in two ways: (1) increasing its permeability for water; (2) altering the methæmoglobin. It is not the change of methæmoglobin into alkaline methæmoglobin which is the important thing, since after washing away the ammonia the brown color is restored and yet the reaction of the corpuscles to water is just the same as if ammonia was still present in sufficient quantity to give the typical red color. One important effect of ammonia is, therefore, the alteration of the permeability of the superficial layer. The relation of the methæmoglobin to the stroma or its solubility may also have been altered.

Some light is thrown on this point by experiments in which blood was rubbed up with sand at various stages of fixation by formaldehyde. It is known that when fresh blood is triturated in this way

laking is produced, apparently because the mechanical changes in the corpuscles permit the entrance of water from the serum. When formaldehyde-fixed blood is rubbed up at a time when laking can still be caused on heating after the addition of ammoniacal water, no evidence can be obtained that any of the blood pigment goes into solution, although the microscope reveals the fragments of numerous broken-down corpuscles. The opportunity has been offered to the serum of dissolving the blood pigment if it were in a condition to enter into aqueous solution. Since it has not done so, we must assume that at this stage it is already insoluble in water or such saline solutions as serum. A part of the ammonia action therefore in laking formaldehyde-fixed corpuscles must be an alteration in the solubility of the blood pigment or in its relation to the stroma. In other words, ammonia not only produces a change in the permeability of the corpuscle, but it unfixes the stroma constituents or the blood pigment.

As to the chemical or physical changes produced by the ammonia, we can assume that in part they are the same as those which lead to the laking of unfixed corpuscles, alkalies being laking agents in virtue of their action on the colloids, forming compounds for example with lecithin¹⁴² and with nucleo-histon.¹⁴³ But in addition we must assume that ammonia has a special action in breaking up compounds which formaldehyde forms with protein. The decidedly smaller effect of sodium hydrate in favoring liberation of the blood pigment from formaldehyde corpuscles speaks in this sense.

F. Blum¹⁴⁴ came to the conclusion that the combinations of formaldehyde with proteins can be considered as methylene derivatives. Benedicenti¹⁴⁵ repeated and extended the observations of Blum, using various proteins and gelatin. He concluded that the formaldehyde-protein compounds are formed by a parallel process to the known reactions of aldehydes on ammonia and the amido-compounds, the point of attack in the protein molecule being the N-containing groups. If aldehydes are brought into contact with ammonia, in general aldehyde-ammonias are

¹⁴² W. Koch, loc. cit.

¹⁴³ For the reactions of nucleohistons and histons which bear on our subject, see Samuely, in Oppenheimer's *Handbuch der Biochemie*, i, p. 308, 1908.

¹⁴⁴ *Anatomischer Anzeiger*, vol. xi, p. 718, 1895; *Zeit. für Physiol. Chem.*, xx, p. 127, 1896.

¹⁴⁵ *Archiv für Anat. u. Physiologie*, p. 219, 1897

formed, a series of compounds which have the empirical composition represented by the addition of a molecule of ammonia to a molecule of aldehyde. These are unstable compounds, and tend easily to either decompose again into their components or to polymerize with loss of water. Thus as the final result of the action of ammonia on formaldehyde we obtain a base usually termed hexamethylenetetramine ($C_6H_{12}N_4$), which, on heating with acids, is easily split into formaldehyde and ammonia. It is partially decomposed into these substances even in neutral watery solutions above $50^\circ C$.¹⁴⁶ Of the reactions of formaldehyde with amido-compounds, the most interesting in connection with our subject is that with urea. This compound easily splits up, giving off formaldehyde.

Schwarz¹⁴⁷ investigated the action of a series of aldehydes, including formaldehyde, on crystallized egg albumin and serum albumin, serum globulin and heteroalbumose, and concluded that the formaldehyde compounds are to be considered as methylene derivatives of the otherwise unaltered protein molecule. Benedicenti¹⁴⁸ found that casein treated with formaldehyde binds less hydrochloric acid after treatment than normal casein. It becomes quite incapable of swelling in hydrochloric acid and indigestible by pepsin or trypsin. But, according to Schwarz, pepsin-hydrochloric acid will digest the formaldehyde-protein compounds studied by him although trypsin will not, the difference being probably due to the hydrochloric acid splitting off the aldehydes and thus freeing the points of attack of the pepsin on the protein molecule. The compounds which the formaldehyde forms with protein can be broken up by steam, all the formaldehyde being recovered on distillation. Gelatin hardened by formaldehyde regains its normal solubility on getting rid of the formaldehyde by steam. The serum-albumin formaldehyde compound is precipitated by various salts, including mercuric chloride (Schwarz). On ammonium salts formaldehyde acts in a different way than on ammonia. On heating formaldehyde and an ammonium salt under pressure, the ammonia is changed successively into mono- di- and trimethylamine¹⁴⁹.

Sollmann¹⁵⁰ has shown that when the alkalinity passes a certain grade

¹⁴⁶ Meyer and Jacobson, *Lehrbuch der Organischen Chemie*, 2d Aufl., Bd. i, p. 749, Leipzig, 1907.

¹⁴⁷ *Zeit. für physiol. Chem.*, xxxi, p. 460, 1900.

¹⁴⁸ *Loc. cit.*

¹⁴⁹ Meyer and Jacobson, *loc. cit.*

¹⁵⁰ *Amer. Journ. of Physiology*, vii, p. 220, 1902.

the precipitate formed by formaldehyde in a weakly alkaline solution of Witte's peptone is redissolved.

Certain of these reactions, if they take place in formaldehyde corpuscles, would help to explain their peculiar behavior. Unfixing of the corpuscle, to the extent that the blood pigment is set free from its combination with the stroma, may be due to the union of the ammonia with the formaldehyde. Solution of the hæmoglobin may be related to the fact that ammonia in the slightest excess dissolves globin. It is known that the presence of salts hinders the laking action of alkalis on unfixed corpuscles. The influence of ammonium salts on ammonia laking is especially great.¹⁵¹ The inhibitory action of ammonium chloride on the laking of formaldehyde corpuscles by ammonia may be accounted for by the hindering effect of ammonium salts on the solution of globin by ammonia. If formaldehyde fixes any constituents of the envelope, the unlocking of these combinations, especially when the temperature is raised, may be expected to increase the permeability of the corpuscle to water, and the presence of excess of water in the interior of the corpuscle which is indicated by its swelling before laking will, of course, favor the solution of blood pigment, which, even after the breaking up of the formaldehyde-protein compounds of the stroma, may still be bound to stroma constituents. In this connection the restoration of the power of imbibition of protein previously treated with formaldehyde when subjected to the action of steam,¹⁵² is of special interest. The favoring action of urea solutions on ammonia laking of formaldehyde corpuscles is to be explained partly by the reaction of urea with formaldehyde mentioned above, partly by the water action of an aqueous solution of a substance like urea which readily penetrates the corpuscles.

In blood partially fixed by formaldehyde and laked by saponin the ghosts are not broken up by water, as is the case in ordinary blood laked by saponin. The explanation is that while saponin still dissolves lipoids in the ghost, it does not dissolve the protein. Therefore the saponin alone does not destroy the ghost. Nor does the water admitted by the saponin action from the serum or from the suspending sodium chloride solution, or water added to the suspending liquid in

¹⁵¹ Arrhenius, *The Chemistry of Immunity*, p. 110, 1907.

¹⁵² Benedicenti, *loc. cit.*

excess, dissolve the nucleo-protein, since it has been sufficiently fixed by formaldehyde. In unfixed corpuscles laked by saponin and then treated with water, the saponin dissolves the lipoids of the ghosts and the water alters the nucleo-protein so that the ghosts disappear.

The laking of sublimate-fixed corpuscles by heating in water to a temperature not higher than that of heat-laking of unfixed corpuscles is an interesting phenomenon. The liberation of the hæmoglobin is rapid and complete. The ghosts remain remarkably distinct and can be filtered off practically without loss. The laking is prevented by hypertonic sodium chloride solution. Mann's conclusion¹⁵³ that "when a protein coagulated by sublimate is treated with a solution of sodium chloride, it becomes soluble" is difficult to understand in view of the fact that sodium chloride prevents the laking of sublimate-fixed corpuscles by heat, unless we assume that the action of the sodium chloride is an osmotic one, water being unable to enter the corpuscle from the sodium chloride solution. Yet, after treatment with 10 per cent sodium chloride solution laking does not take place even on heating with distilled water, possibly because the sodium chloride has precipitated nucleo-histon. Sodium chloride precipitates neutral salts of histon, while mercuric chloride does not precipitate histons.¹⁵⁴ In the corpuscles, some of whose constituents have been fixed by mercuric chloride, the additional action of sodium chloride may be sufficient to determine a change which prevents subsequent laking, although the sodium chloride itself does not prevent the laking of normal corpuscles. Laking of a watery suspension of sublimate-fixed corpuscles by amyl alcohol is also prevented by treatment with 0.9 per cent sodium chloride solution. After the action of 10 per cent sodium chloride, even ammonium sulphide will not lake sublimate corpuscles.

If, at the stage of sublimate fixation where laking is readily produced by heating in water at 55° C., the fixed corpuscles suspended in water be rubbed up with sand at room temperature, no blood pigment goes into solution, although the corpuscles are seen under the microscope to have been broken down in large numbers to a granular débris. In the interior of the corpuscle, then, at this stage none of the pigment is in a water-soluble state. Yet it can be shown by spectroscopic

¹⁵³ Chemistry of the Proteids, p. 313.

¹⁵⁴ Oppenheimer's Handbuch, loc. cit,

examination that much of it is still so little altered that it gives the oxyhæmoglobin bands. This is illustrated by the following excerpt from the protocols:

March 2, 1909. Added to ox-blood an equal volume of 4 per cent formaldehyde solution (in 0.9 per cent sodium chloride). To another portion of the blood added one-fourth of its volume of a saturated aqueous solution of mercuric chloride. Kept in cool room.

March 4. Centrifuged some of the sublimate blood, which has a brown color, and a spectrum with a good (methæmoglobin) band in the red and two strong oxyhæmoglobin bands. Some blood pigment is in solution in the superjacent liquid. This liquid shows a spectrum with a strong band in the red in position of methæmoglobin band and two fainter bands in position of oxyhæmoglobin bands. On addition of ammonia to the liquid the band in the red disappears, and the band of alkaline methæmoglobin just to the left of D appears. The two other bands persist. Added ammonia to some of the sublimate blood. It laves immediately, and then shows no band in the red. The oxyhæmoglobin bands remain unchanged and very strong, and there is a fainter band just to the left of D. On comparison of the spectra of the superjacent liquid and the laked blood after addition of ammonia, the following differences are noted: (1) When the two liquids are made of approximately equal strength the oxyhæmoglobin bands are much stronger in the laked blood. (2) On diluting the laked blood till the tint is considerably weaker than that of the superjacent liquid, the band immediately to the left of D disappears, while the oxyhæmoglobin bands are still quite markedly stronger than in the superjacent liquid. (3) The tint of the diluted superjacent liquid when held against the sky has a distinct brownish element in it; that of the diluted laked blood is much more nearly a pure red. The explanation probably is that in the interior of the corpuscles there is still much unaltered blood pigment, or at any rate blood pigment so little altered that it still gives the oxyhæmoglobin spectrum, even if it is united in some way to the mercury salt. The blood pigment liberated in the superjacent liquid is of course more profoundly altered by the sublimate. The brown color of the corpuscles is probably due to the alteration of the pigment in the more superficial portions of the corpuscle. That the bands in the position of the oxyhæmoglobin bands are not those of so-called alkaline methæmoglobin was shown by observing the spectrum, while ammonia was pipetted on to the blood in the parallel-sided trough. No change took place in the bands.

The most probable conclusion is that heating causes a change in some of the other constituents of the corpuscle than the blood pigment, which permits the pigment to go into solution and escape. This change is probably not essentially different from that which takes place in the heat laking of ordinary corpuscles. The common statement¹⁵⁵ is that mercuric chloride does not precipitate oxyhæmoglobin till the oxyhæmoglobin is decomposed and has become brown owing to the formation of hæmatin. If this is correct we must assume that in the partially fixed sublimate corpuscles, at the stage mentioned, the undecomposed oxyhæmoglobin is prevented from going into solution when the corpuscles are triturated with water because the mercury salt has fixed some constituents of the stroma. Either the precipitation of these constituents throughout the whole substance of the stroma mechanically prevents the water from dissolving the oxyhæmoglobin, which seems quite improbable, or the mercuric chloride, by uniting with the stroma constituent of the hæmochrome complex, renders the hæmochrome insoluble to water without essentially altering its spectrum.

Permeability of the nucleus and its relation to hæmolysis. The nuclear membrane and the envelope of the corpuscle have each their special properties. They are not in general affected equally nor in the same sense by a given agent. Great swelling of the nucleus can be produced while the corpuscles remain of approximately normal size, and great swelling of the corpuscle without marked change in the size of the nucleus. The fact that methylene blue stains the nuclear membrane as a rule more deeply than the envelope of the corpuscle suggests that the quantity of protein in the former is greater. Bing and Ellermann¹⁵⁶ have shown that in the staining of the medullary sheath with methylene blue, the absence of the lipoids does not affect the result.

The absence of swelling of the nuclei of *Necturus* corpuscles fixed by formaldehyde and then laked by ammonia perhaps indicates that the nucleus or the nuclear membrane is richer in nucleo-histons and less rich in lipoids than the envelope and the stroma of the corpuscle. The same explanation may be given for the osmic acid corpuscles, which swell on heating in ammoniacal water, while the nucleus does not.

¹⁵⁵ Gamgee in Schäfer's Text-book of Physiology, i, p. 207.

¹⁵⁶ Archiv für Anat. u. Physiol., p. 256, 1901.

On the other hand, the Necturus corpuscles fixed by Hayem's solution when laked by water and ammonia show swelling of the nucleus, possibly because ammonium chloride does not precipitate histon¹⁵⁷ so that the nucleus is in a position to swell on addition of ammonia. Ammonium salts of nucleo-histons are soluble in water, and therefore the nuclear membrane after the action of ammonia admits water easily. Sulphuretted hydrogen added to sublimate corpuscles causes laking at the ordinary temperature without swelling of the nucleus or change in the intranuclear network. Therefore, the constituents of the nucleus or of the nuclear membrane which has been acted upon by the mercury salt are either more firmly fixed by it than the constituents of the corpuscular envelope and stroma, or the breaking up of their mercury compounds leaves them relatively unaffected as regards their osmotic properties. The fact that sapotoxin does not cause swelling of the nucleus when it lakes Necturus corpuscles, although it removes some of the stain from the nuclei of heat-laked ghosts stained with methylene blue also suggests that lipoids are not so important in the nuclear membrane as in the cell envelope,

Sodium taurocholate does not at first swell the nuclei of Necturus corpuscles, although later on they become swollen, possibly by a water action owing to the great increase in water in the contents of the corpuscle. This conclusion is supported by the fact that urea solution (1 per cent) causes swelling of the nucleus as well as of the corpuscle, doubtless because urea easily penetrates both, and water itself has the same effect. The taurocholate usually does not cause swelling of the corpuscles, in which case the excess of water in the corpuscles necessary for the swelling of the nucleus does not exist, unless the blood pigment has been already discharged and its place occupied by water.

It is unnecessary to labor the point that the permeability of the nucleus is different from that of the corpuscle. For example, ammonium chloride is taken up greedily by the extranuclear portion of the hen's corpuscle, but not by the nucleus.¹⁵⁸ This difference is certainly due in part to a difference in the properties of the nuclear and corpuscular envelopes. But it may be partly due to a difference in the contents of the two structures.

¹⁵⁷ Samuely, loc. cit.

¹⁵⁸ Amer. Journ. of Physiol., viii, p. 107, 1902.

Classification of laking agents. This paper is concerned with the more general aspects of hæmolysis and does not deal in detail with all the very numerous laking agents. Certain points, however, in connection with the mechanism of laking by some of the groups into which hæmolytic agents can be divided, may now be summarized.

Laking may be brought about by (1) mechanical means (pressure, trituration, shaking); (2) physical changes (freezing and thawing, heat, condensor discharges, water, drying and subsequent exposure to salt solutions); (3) chemical agents (saponin, bile salts, ether, chloroform, acids, alkalies, etc.); (4) biological agents (the specific hæmolysins, bacterial hæmolysins—including the bodies active in putrefactive hæmolysis, spontaneous laking, autolysis).

This classification is not a strict one, although it is useful for purposes of description. It is quite probable, for example, that interchange of water between the corpuscles and the suspending liquid is produced by the first group and that chemical changes are produced by the second. On the other hand, physical effects are unquestionably concerned in the action of such reagents as saponin, which dissolves cholesterin and lecithin. In biological hæmolysis properly so called both chemical and physical reactions are also in all probability concerned.

Mechanical Laking. Possibly the laking produced by shaking is due to a precipitaton of some of the colloids of the corpuscles like the precipitation of albumin and other proteins studied by Ramsden. The laking produced by rubbing up with sand may be due to the alteration of the envelope or stroma in such a way as to permit the entrance of some water into the corpuscle. Yet it differs from the ordinary water laking in this, that the conductivity of the laked blood is but little increased, and the action of saponin on the ghosts causes a great further increase of conductivity. Another factor in the laking by trituration may be mechanical squeezing of a certain amount of water out of the corpuscles, leading to a more labile condition of the blood pigment in them analogous to that observed by Peskind,¹⁵⁹ who saw that after the precipitation of corpuscles by slight excess of acid salts (ammonium ferrous sulphate) pressure on the cover slip caused the blood pigment

¹⁵⁹ Amer. Journ. of Physiol., viii, p. 411, 1903.

to pass out, leaving almost colorless shadows. The intraglobular crystallization produced by abstraction of water from the corpuscles of fishes' blood by hypertonic solutions of salts¹⁶⁰ is perhaps a similar phenomenon.

The conclusion that the amount of trituration necessary to liberate all the blood pigment does not alter the stroma so profoundly as water does is corroborated by an observation of Belonowski.¹⁶¹ Working with the spider poison (arachnolysin) he found that the stromata of corpuscles of a species sensitive to the toxin, after laking by rubbing up the blood with sand and decanting off the serum, have a much greater binding power for the toxin than stromata obtained by the method of Sachs (heating to the temperature of heat-laking and treatment with 6 to 10 volumes of water) or by the method of Pascucci, in which treatment with excess of water is also a step.

Heat-Laking—Reasons have already been given why this cannot be considered as due merely to the melting of fatty constituents of the corpuscles at a definite temperature. There is every reason to believe, however, that combinations containing lipoids are disrupted under the favoring influence of heat once a certain critical temperature has been passed. The stromata do not entirely lose their semi-permeable properties, as they do if the temperature is raised to the coagulation point of the serum proteins. Nor in careful heat laking are the electrolytes liberated from the stromata in such quantity as in violent methods of laking like saponin or water laking. Laking by condenser discharges, which according to Rollett¹⁶² does not cause liberation of the electrolytes along with the hæmoglobin, may very well be a form of heat laking, as Cremer¹⁶³ suggests, due to local heating of the badly conducting envelopes of the corpuscles.

Laking of Dried Corpuscles.—After the loss of a certain amount of water, as has been shown by me,¹⁶⁴ and in greater detail by my pupil, Prof. C. C. Guthrie,¹⁶⁵ the erythrocytes are laked by isotonic or even

¹⁶⁰ Hamburger, Osmotischer Druck I.

¹⁶¹ Bio-chem. Zeit., v, p. 65, 1907.

¹⁶² Pflüger's Archiv, lxxxii, p. 199, 1900.

¹⁶³ Zeit. für Biol., xvi, p. 101, 1904.

¹⁶⁴ American Journ. of Physiology, viii, p. 117, 1902.

¹⁶⁵ Ibid., p. 441, 1903.

by hypotonic salt solutions, and by their own serum. The permeability of the corpuscles is radically altered by drying at air temperature, so that dried corpuscles are far less normal in their osmotic relations than formaldehyde-fixed corpuscles. Doubtless this is a reason why corpuscles fixed by any process in which drying of smears is a step stain better than moist corpuscles. Heat fixation causes also a great increase in the permeability. It follows from this that fixed corpuscles which have been subjected to drying or heating before fixation are unsuitable for experiments on permeability, although they have sometimes been employed, for instance in the study of the permeability of the corpuscles for dyes. It is easy to see that the loss of a certain amount of water may upset the equilibrium of the colloid solutions in the corpuscles, leading it may be to precipitation of proteins and other colloids in the envelope or in the interior of the stroma.

Laking by Chemical Agents.—A noteworthy circumstance in laking by these agents is that a certain amount, even of those which very easily penetrate the corpuscles, must be taken up before any hæmoglobin whatever is liberated. Thus ethyl alcohol, dissolved to the extent of 10 per cent by volume in 1 per cent sodium chloride solution, does not cause the least laking in twenty-four hours in the cold, although the blood is still rapidly laked by addition of more alcohol or by water.¹⁶⁶

Laking by Foreign Serum.—According to Arrhenius¹⁶⁷ the immune body (amboceptor) is not chemically bound by the erythrocytes. Perhaps it is taken up by a surface action (adsorption). For, (1), like saponin, it is readily taken up at 0° C., that is, the temperature coefficient is small. Now, saponin produces at 0° C. almost as great an increase in the conductivity of formaldehyde corpuscles as at ordinary temperature, and therefore its action may be supposed to be largely a surface action. (2) The amboceptor is relatively thermostable, and so is the agglutinin, which certainly has a surface action as a fundamental part of its effect. (3) As already mentioned, substances which give rise to the production of specific hæmolysins and agglutinins are still present in the active condition in formaldehyde corpuscles. Now, according to Bang and Forssman¹⁶⁸ the hæmoly-

¹⁶⁶ Journ. of Med. Research., loc. cit., p. 304.

¹⁶⁷ Chemistry of Immunity, 1907.

¹⁶⁸ Hofmeister's Beiträge, viii, p. 238, 1906.

sinogens are substances which can be extracted by ether and similar solvents, and we know that the extraction of ether-soluble substances from formaldehyde corpuscles produces a change in the surface layer. Therefore, probably hæmolysinogenic substance (antigen) is present in the surface layer of the corpuscle. So that if the substance in the corpuscle which fixes hæmolysin (amboceptor) is identical with hæmolysinogen or related to it, the amboceptor action might be expected to be an action on the surface of the corpuscle. (4) The surface being altered by the amboceptor, the complement may gain admittance by some reaction with a much greater temperature coefficient than that concerned in taking up the amboceptor, a reaction perhaps due to ordinary chemical affinity. The permeability of the corpuscle to water need not be materially increased by the amboceptor (absence of laking in corpuscles charged with amboceptor alone).

The complement when fixed may determine the increase of permeability to water and also the loosening of the bonds between the stroma and the hæmochrome. Instead of activation of the complement by amboceptor in the serum, the amboceptor at the temperature at which laking takes place may be rapidly adsorbed, and the complement more slowly, giving rise to gradual laking. Arrhenius believes that a union of amboceptor and complement takes place in the interior of the corpuscle. Yet that some change occurs in the permeability of corpuscles under the influence of amboceptor alone is indicated by the fact that the conductivity of rabbit's blood treated with dog's serum after inactivation by heat is not the same as it would have been had a similar quantity of an indifferent salt solution been added to the blood instead of the dog's serum, but is distinctly less than this. It is interesting that in blood artificially enriched with ammonium chloride, foreign serum inactivated by heating to 60° produces a change in conductivity in the same sense as the unheated serum, although not so marked, that is, in the opposite sense to the change produced by foreign serum in the case of normal blood. Thus in Table X¹⁶⁹ the conductivity of the mixture of heated dog's serum and ammonium chloride rabbit's blood was 101.4, whereas, if simple mixture had taken place it would have only been 91.5. The same explanation may be given of the difference as on page 78.

¹⁶⁹ Journ. of Physiol., xxiv, p. 228.

In this connection we might think of J. Traube's statement,¹⁷⁰ that the surface tension of serum is diminished by heating to 56° by the formation of substances which are adsorbed or dissolved in lipoids in the presence of red corpuscles. He suggests that in this way the envelopes of the corpuscles are thickened and that this thickening hinders hæmolysis, although it does not hinder the attachment of the amboceptor. This idea, however, does not explain the phenomenon we have been discussing. For although a corpuscle with a thickened envelope might be expected to be even less permeable to serum ions than a normal corpuscle, the normal corpuscle is such a bad conductor that no possible diminution in its conductivity could produce so distinct a diminution in the conductivity of the suspension as was observed. It is possible that the agglutinin plays some part in this change. Even in the case of so energetic a laking agent as water, one has observed that the agglutination produced by it need not entail the entrance of water and consequent swelling of corpuscles. Crenated corpuscles can agglutinate. Laking by water, however, is always preceded by swelling of the corpuscles.

Possibly the substances responsible for Traube's phenomenon are the same as cause the increase of alkalinity of serum inactivated by heat observed by Liebermann¹⁷¹ and confirmed by Seligmann.¹⁷²

The Influence of Cane-sugar on Serum Laking.—It has been shown that this form of hæmolysis is inhibited by cane-sugar. What is the mechanism of this action? In one experiment¹⁷³ a cane-sugar solution slightly hypertonic was used. This would cause slight dilution of the serum and therefore a small diminution of its specific conductivity. In the case of the heated serum the conductivity of the liquid removed after action on the rabbit's corpuscles was 49.13. The conductivity of the liquid calculated on the assumption that simple mixture had taken place is 50.3. No hæmoglobin was in solution. For the blood mixed with heated serum the conductivity was exactly the same as the calculated conductivity. This agrees well with the assumption that in presence of cane-sugar no change occurred in the distribution of

¹⁷⁰ Bio-chem. Zeit., x, p. 380, 1908.

¹⁷¹ Archiv für Hygiene, lxii, 1907.

¹⁷² Bio-chem. Zeit., x, p. 430, 1908.

¹⁷³ Journ. of Med. Research, viii, p. 302.

serum ions. The slight diminution of conductivity of the extra-corpuscular liquid would be made up in the case of the blood by a widening of the conduction paths through the slight shrinking of the corpuscles. Either then the amboceptor is not fixed by the corpuscles in the presence of cane-sugar, which is negatived by the fact that with unheated serum some laking occurs, or the fixing of the amboceptor does not produce that change in the corpuscles in the presence of sugar which permits water and electrolytes to enter them, as occurs in the case of dog's serum (unheated and heated) acting on rabbit's corpuscles in the absence of sugar. It is easy to see that the presence of sugar which could not enter the corpuscles might prevent water and salts from entering them. For if we suppose that the corpuscles and serum are in equilibrium at the outset, and that the amboceptor renders the envelope more permeable to salts, the salt must in entering the corpuscles be accompanied by a corresponding amount of water. Otherwise the osmotic pressure in the corpuscles would be increased and that outside the corpuscles diminished. The difference of pressure would bring the entrance of salts into the corpuscles to an end, or water must accompany them.

Spontaneous Laking.—The so-called spontaneous laking, *i.e.*, the laking which takes place when blood is preserved in sterile tubes (or in the cavities of sterile paraffine) is possibly an autolytic process¹⁷⁴ due to ferments developed in the erythrocytes themselves, or to ferments derived from the leucocytes or the serum, or to the action of bodies produced by the autolysis of the serum proteins or other constituents of the blood. The conductivity and Δ of the laked blood, at first altered but little, are ultimately increased very greatly, although far less than when putrefaction is allowed to take place.¹⁷⁵ Precisely similar observations have since been published by Oker Blom,¹⁷⁶ by Buglia¹⁷⁷ and by Polimanti.¹⁷⁸

¹⁷⁴ Stewart, Amer. Journ. of Phys., xi, p. 374, 1904.

¹⁷⁵ Stewart, Journ. of Exp. Med., iv, p. 235, 1899.

¹⁷⁶ Skand: Archiv, xiv, p. 48, 1903.

¹⁷⁷ Archivio di Fisiologia, iv, p. 56, 1906.

¹⁷⁸ Bio-chem. Zeit., xi, p. 260, 1908.

